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THE ULTRACENTRIFUGAL COMPOSITION OF NORMAL RABBIT SERUM

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(Received for publication, August 3, 1949)

Studies reported in the literature (1, 2) have indicated the presence of a density-sensitive lipoprotein capable of giving rise to asymmetrical albumin peaks in the ultracentrifugal diagram only in the serum of humans. In the present study of twenty-two rabbit sera, a density-sensitive component giving rise to asymmetry of the albumin peak has definitely been observed in thirteen cases.

EXPERIMENTAL

All analytical runs were made with an electrically driven ultracentrifuge designed by Pickels (3) (Spino model E). A rotor speed of 59,780 R.P.M. was maintained, giving centrifugal forces of 240,000 to 300,000 $\times g$ at the inside and outside of the centrifugal field, respectively. Blood was drawn by venipuncture of the rabbit ear and allowed to clot for periods of 2 to 3 hours at room temperature before preparation of the serum. Serum thus obtained was stored at 0-4° until ultracentrifugation.

Ultracentrifugal runs were made on twenty-two sera in the unaltered state. In all cases, components corresponding to the so called "20" component, "globulin," and "albumin" were observed. Thirteen rabbit sera demonstrated asymmetry of the albumin peak, of variable degree, whereas the other nine sera showed symmetrical "albumin" peaks in the ultracentrifugal diagrams. Fig. 1, *a*, *b*, and *c* shows typical photographs of the asymmetrical albumin peaks, whereas Fig. 1, *d* shows a typical symmetrical "albumin" peak. Fig. 1 shows that the degree of asymmetry is quite variable among those samples in which asymmetry was demonstrated. The similarity of these diagrams to those obtainable in human sera, in which the albumin asymmetry is associated with the presence of a density-sensitive lipoprotein, is striking. It was therefore of interest to determine the nature of the component of rabbit serum responsible for this phenomenon. Studies of the variation in observed patterns with alteration in density of the serum samples were then made. Density increments were produced by the addition of small quantities of either sucrose or sodium chloride to the serum. When this was done to the serum samples which gave rise to asymmetrical albumin peaks in the

unaltered state, the albumin peak became symmetrical and an "inverse" peak, rapidly moving toward the inside of the centrifugal field, was observed. Moreover, for a given serum sample the area under the "inverse" peak observed in such flotation studies was related to the degree of albumin boundary asymmetry obtained with runs on the unaltered serum sample.

We interpret the asymmetry of the albumin peak in the ultracentrifugal diagram to be caused by a piling up of a density-sensitive component (specific volume ~ 0.97) on the albumin concentration gradient. A detailed analysis of this pile up phenomenon is given by Gofman, Lindgren, and Elliott (4) in their study of human sera.

Direct confirmatory evidence for the pile up phenomenon has been obtained in the present studies by use of the angle preparative rotor. Since no difficulties in formation or maintenance of boundaries were experienced

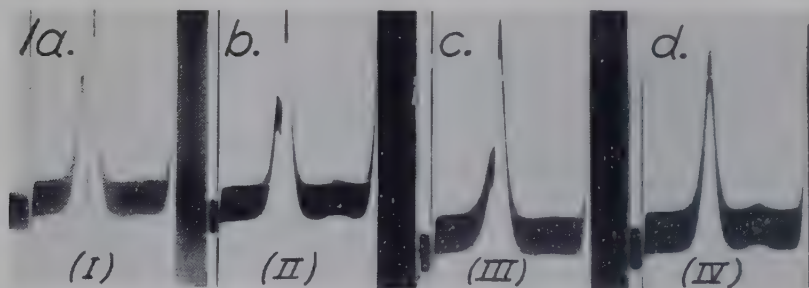


FIG. 1. Ultracentrifugal patterns of unaltered rabbit serum, Samples I to IV, obtained approximately $2\frac{1}{2}$ hours after the rotor had attained full speed. Bar angles were 65° except for *c* and *d*, which were 60° and 55° respectively. The cell used had a capacity of 0.3 ml.

in these studies, it appears that the conditions for successful experiments with the angle centrifuge, as outlined by Pickels (5), were fulfilled. Samples of rabbit serum run unaltered in the angle centrifuge rotor for a sufficient time revealed an intense Tyndall scattering band on the albumin concentration gradient. Intensity of Tyndall scattering increased with increasing albumin boundary asymmetry as observed in the analytical runs. Also, the addition of a sufficient density increment to the serum sample followed by a run in the angle centrifuge revealed no Tyndall scattering from the albumin boundary. All unaltered rabbit serum samples studied with the angle centrifuge demonstrated the Tyndall scattering from the albumin boundary.¹ Thus, this was true even of those samples of rabbit serum in which the concentration of this density-sensitive component was so low that its presence was not discernible either by albumin boundary asymmetry or by an inverse peak in an analytical flotation

¹ Similar Tyndall scattering from the albumin boundary occurs in human serum, heifer serum, and rabbit lymph.

run. Figs. 2, *a* and 2, *b* show photographs of Tyndall scatterins from the albumin boundary of unaltered serum Samples III and IV respectively,

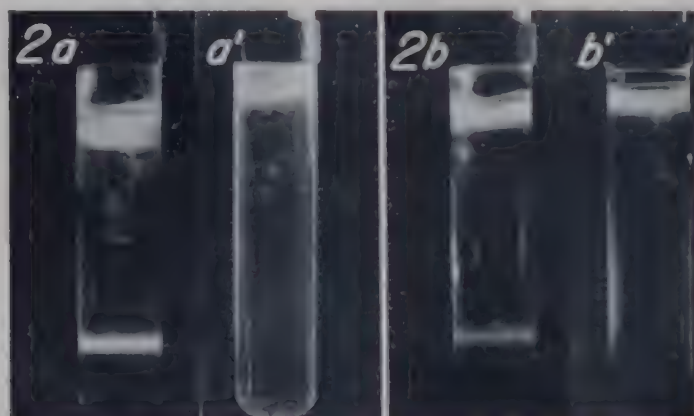


FIG. 2. Photographs of Tyndall scattering obtained at 90° to the collimated light source. Fig. 2, *a* and *b* are serum Samples III and IV respectively, whereas Fig. 2, *a'* and *b'* are Samples III and IV with an appropriate density increment. Tyndall scattering can be observed at the top of all the preparative centrifuge tubes because of the presence in rabbit sera of lipides or lipoproteins of large particle size, whose densities are less than that of the serum supernatant

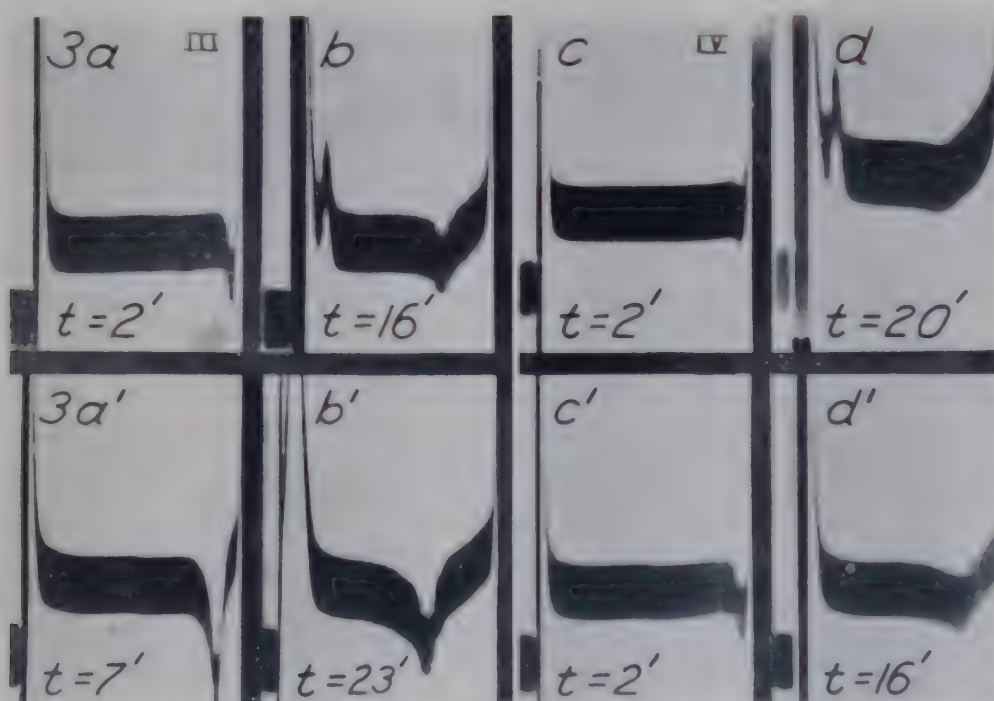


FIG. 3. *a*, *b*, *c*, and *d*, flotation of the density sensitive component in serum Samples III and IV. *a'*, *b'*, *c'*, and *d'*, flotation of this component in the corresponding concentrated samples obtained with the angle centrifuge. Bar angles all 45° except for *d* which was 35° . The cell used had capacity of 0.8 ml. *t* = time in minutes after the rotor attained full speed.

whereas Figs. 2, *a'* and 2, *b'* show the absence of this Tyndall scattering in Samples III and IV with an appropriate density increment.

Experiments were then performed which indicated that the pile up is

complete after a sufficient time in the ultracentrifugation of unaltered serum. A measure of the concentration of the density-sensitive component in Samples III and IV of rabbit serum was obtained by measurement of the area under the inverse peak during flotation of this component (Fig. 3, *a*, *b*, *c*, and *d*). Additional serum from Samples III and IV was then run unaltered in the angle centrifuge and the density-sensitive component pipetted from the albumin boundary. Since the preparative centrifuge tubes contained 6 ml. of serum Samples III and IV respectively and the final volume of the pipetted samples was 2 ml., concentration of this component should have been increased by a factor of 3 in each case, assuming complete pile up. Analytical flotation measurements of the concentrated samples (Fig. 3, *a'*, *b'*, *c'*, and *d'*) gave a factor of increased concentration of 2.4 and 3.7. This agreement within the experimental error of measurements of small areas provides evidence of complete pile up.

The actual concentration of this component in serum Sample III (demonstrating the asymmetry of Fig. 1, *c*) was 0.09 per cent, assuming a specific refractive increment² equal to that of the β_1 -lipoprotein of human serum. Chemical analysis of this component obtained by pipetting it from the albumin boundary showed that a large fraction of the free and esterified serum cholesterol is carried by this component.

SUMMARY

A density-sensitive component present in normal rabbit serum reveals itself in approximately 60 per cent of the sera studied by producing asymmetry of the albumin boundary. In the remaining sera, the concentration of this component is too low to produce distortion of the albumin boundary but its presence can be demonstrated by studies of light scattering with the angle centrifuge. Further, the evidence obtained with light-scattering experiments affords direct confirmation of the pile up theory (4).

BIBLIOGRAPHY

1. Pedersen, K. O., *J. Phys. and Colloid Chem.*, **51**, 156 (1947).
2. Edsall, J. T., *Advances in Protein Chem.*, **3**, 459 (1947).
3. Pickels, E. G., *Chem. Rev.*, **30**, 341 (1942).
4. Gofman, J. W., Lindgren, F. T., and Elliott, H., *J. Biol. Chem.*, **179**, 973 (1949).
5. Pickels, E. G., *J. Gen. Physiol.*, **26**, 341 (1943).
6. Armstrong, S. H., Jr., Budka, M. J. E., Morrison, K. C., and Hasson, M., *J. Am. Chem. Soc.*, **69**, 1747 (1947).

² $\Delta n = 0.00171$ expressed as the refractive increment per gm. per 100 ml. of aqueous solution as given by Armstrong *et al.* (6).

THE METABOLISM OF INTRAVENOUSLY ADMINISTERED AMMONIUM GLUTAMATE AND GLUTAMINE

BY MAX BERENBOM* AND JULIUS WHITE

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(Received for publication, May 21, 1949)

In recent years much knowledge has been accumulated concerning the metabolism of N^{15} -labeled compounds when administered orally, while only limited information is available with regard to their fate when injected directly into the blood stream. To obtain further information concerning the fate of certain groups of compounds when administered parenterally, studies have been initiated on the metabolism of N^{15} -labeled compounds administered intravenously. Friedberg *et al.* (1) and Awapara and Marvin (2) have shown that intravenously administered amino acids are rapidly removed from the blood stream and distributed in various tissues of the body. It was also demonstrated (2) that, within a period of 15 minutes after the injection of certain amino acids, conversion to other amino acids had occurred. The purpose of the present investigation is to study the metabolism of intravenously administered ammonium glutamate labeled with N^{15} in the ammonium ion and glutamine labeled with N^{15} in the amide group.

Studies with N^{15} -labeled ammonium citrate have shown that orally administered ammonia is incorporated into body proteins (3-5) and that the percentage of N^{15} excreted in the urine is dependent on the level of protein intake (5).

Studies on glutamine have been extensive (6) and much information has been accumulated concerning its metabolism. Studies *in vitro* have shown that a wide variety of tissues contain enzymes that can synthesize (7, 8) or desamidate glutamine (9, 10). The presence of glutamine has been demonstrated in blood and other fluids (11-15). Van Slyke *et al.* (16) have shown that plasma glutamine is the precursor of a large fraction of the urinary ammonia formed in the kidneys of acidotic dogs. This was the first demonstration *in vivo* of a physiological function of glutamine in the animal kingdom. Experiments with liver slices in several species of animals have shown that glutamine forms urea (17-20), probably by a mechanism which is independent of the Krebs ornithine system (6). More recently Leuthardt (21) has shown that glutamine administered orally to guinea pigs causes an increase in urinary urea.

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Data are herein reported on the production of urea from intravenously administered ammonium glutamate and glutamine each labeled with N^{15} .

EXPERIMENTAL

Isotopic Ammonium Glutamate—Isotopic ammonium glutamate was prepared by steam distilling isotopic ammonia (14.6 atom per cent excess of N^{15}) into an excess of glutamic acid suspended in water. The excess of glutamic acid was removed by filtration and the solution of ammonium glutamate (14.6 atom per cent excess of N^{15}) was made up to a known volume.

Isotopic Glutamine—Glutamine containing N^{15} in the amide group was synthesized according to the method of Bergmann, Zervas, and Salzmann (22) with precautions to recover the unchanged isotope. The yield of glutamine, m.p. 186–187° (uncorrected), $[\alpha]_D^{23} = +6.2^\circ$ (2 per cent in water),¹ was 35 per cent based on the ammonia used and contained 14.3 atom per cent excess of N^{15} in the amide group. By use of Archibald's critique (6) for purity, an aqueous solution of glutamine when treated with Nessler's reagent gave no immediate color, indicating the absence of ammonium pyrrolidone carboxylate.

$C_5H_{10}N_2O_3$.	Calculated.	C 41.1,	H 6.9,	N 19.2,	amide N 9.6
	Found. ²	" 40.6,	" 7.0,	" 18.6,	" " 9.3

General Procedure—Stock male rats of the Osborne-Mendel strain, which weighed between 200 and 230 gm., were used. In one series of animals an aqueous solution of glutamine (14.3 atom per cent excess of N^{15} in the amide group) equivalent to 4.75 mg. of amide nitrogen per animal was injected into the jugular vein; in another series of animals an aqueous solution of ammonium glutamate (14.6 atom per cent excess of N^{15} in the ammonium group) equivalent to 5.0 mg. of ammonia nitrogen per animal was similarly injected. The animals were kept in individual metabolism cages and were supplied with a stock diet of the following percentage composition: sucrose 60, Crisco 19, casein 12, salts 4,³ corn oil 5. This was supplemented with the following amounts of vitamins per kilo of diet: thiamine hydrochloride 3 mg., riboflavin 2 mg., pyridoxine hydrochloride 2.5 mg., calcium pantothenate 7 mg., choline hydrochloride 30 mg. Water was supplied *ad libitum*. At intervals of 15 minutes, 1, 6, and 24 hours respectively, four animals from each series were placed under light ether anesthesia, injected with heparin, and exsanguinated from the internal carotid artery. The blood was collected, and the livers and kidneys were

¹ This value for the specific rotation of glutamine agrees with that obtained by Fruton (23) using the procedure of Bergmann *et al.* (22).

² Analyses for C, H, and N were made by Mr. R. J. Koegel.

³ Hubbel, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutr.*, **14**, 273 (1937).

excised and pooled respectively. The urine was collected (except in the 15 minute experiment), pooled, and made up to a known volume. The blood, livers, and kidneys were hydrolyzed by refluxing with 20 per cent hydrochloric acid for 18 hours, and were then treated with charcoal, filtered, and evaporated to dryness under reduced pressure. The concen-

TABLE I

*Distribution of N^{15} in Rats Following Intravenous Injection of $N^{15}H_4$ Glutamate or Glutamine Containing N^{15} in Amide Group**

Material analyzed	Time	Distribution of nitrogen					
		Total N, m.eq.		N^{15} concentration, atom per cent excess†		Per cent recovery of injected N^{15}	
		Glutamine	NH_4 glutamate	Glutamine	NH_4 glutamate	Glutamine	NH_4 glutamate
	<i>hrs.</i>						
Liver	0.25	62.4	77.0	0.042	0.029	13.5	10.7
	1	84.9	83.9	0.021	0.025	9.2	10.1
	6	60.4	54.8	0.019	0.017	5.9	4.5
	24	61.1	68.4	0.016	0.014	5.1	4.6
Blood	0.25	65.1	62.5	0.012	0.014	4.7	4.2
	1	53.9	72.3	0.011	0.010	3.1	3.5
	6	49.7	56.8	0.010	0.010	2.6	2.7
	24	58.7	61.7	0.008	0.009	2.4	2.6
Kidney	0.25	11.3	10.4	0.032	0.049	1.9	2.5
	1	13.7	12.6	0.023	0.032	1.6	1.9
	6	11.9	11.1	0.018	0.022	1.1	1.2
	24	11.0	12.4	0.014	0.012	0.8	0.7

* Each value represents the analysis of a pooled sample from four rats receiving a total of 19 mg. of amide nitrogen (14.3 atom per cent excess of N^{15}) in the experiments with glutamine and 20 mg. of ammonia nitrogen (14.6 atom per cent excess of N^{15}) in the experiments with NH_4 glutamate.

† Analysis of non-isotopic ammonium sulfate gave values of 0.0045 ± 0.0005 atom per cent excess of N^{15} .

trates were dissolved in water and made up to a known volume. Aliquots of these solutions were analyzed for total nitrogen (Kjeldahl) and N^{15} .⁴

Urine—Urea was isolated quantitatively from the urine as dioxanthylurea and its N^{15} concentration determined. The N^{15} content of urinary ammonia was also determined.

RESULTS AND DISCUSSION

The results show (Tables I and II) that intravenously administered ammonium glutamate and glutamine, respectively, were rapidly removed

⁴ Determinations for N^{15} were made on a Consolidated Nier isotope-ratio mass spectrometer.

from the blood stream of the rat, converted to urea, and excreted in the urine. 66 per cent of the injected isotope was excreted in the urine in 24 hours in each instance. During this period 94 per cent of the excreted N^{15} (62 per cent of the total dose) derived from injected glutamine was accounted for as urea while with ammonium glutamate 80 per cent of the excreted N^{15} (53 per cent of the total dose) was accounted for as urea. The excretion of N^{15} in the urine of rats following intravenous administration of ammonium glutamate or glutamine was greater than that following the oral administration of labeled amino acids to rats maintained on a normal protein diet and approximately the same as that following the oral

TABLE II

*Distribution of N^{15} in Urine of Rats Following Intravenous Injection of $N^{15}H_4$ Glutamate or Glutamine Containing N^{15} in Amide Group**

Analysis	Time	Total N, m.eq.		N^{15} concentration, atom per cent excess		Per cent recovery of injected N^{15}	
		Glutamine	NH_4 glutamate	Glutamine	NH_4 glutamate	Glutamine	NH_4 glutamate
	<i>hrs.</i>						
Total nitrogen	1	4.9	3.3	0.495	0.615	12.5	9.7
	6	19.3	20.6	0.381	0.543	38.1	50.1
	24	88.6	58.3	0.144	0.237	66.0	66.1
Urea	1	4.4	2.3	0.428	0.436	9.6	4.8
	6	16.6	15.9	0.402	0.537	34.4	40.8
	24	78.0	47.1	0.154	0.234	62.0	52.7
Ammonia	1	0.27	0.32	1.962	2.62	2.7	4.1
	6	1.84	1.27	0.379	1.44	3.6	8.9
	24	4.0	2.65	0.205	0.809	4.2	10.3

* Each value represents the analysis of a pooled sample from four rats receiving a total of 19 mg. of amide nitrogen (14.3 atom per cent excess of N^{15}) in the experiments with glutamine and 20 mg. of ammonia nitrogen (14.6 atom per cent excess N^{15}) in the experiments with NH_4 glutamate.

administration of labeled amino acids or ammonia to rats on a high and normal protein diet respectively (5).

In spite of the rapid conversion of injected isotopic ammonium glutamate and glutamine to urea there was no great accumulation of isotopic nitrogen in the liver and kidney. 15 minutes after the intravenous administration of isotopic glutamine and ammonium glutamate respectively, 14 per cent of the N^{15} of the former and 11 per cent of the latter were found in the liver. However, of the total amount of isotope remaining in the body after 15 minutes, 1, 6, or 24 hours approximately the same percentage was found in the liver (Table III). This seems to indicate that nitrogen is continuously supplied to the liver where it is rapidly converted

to urea. Rittenberg and Shemin (24) have reported similar conclusions from observations after oral administration of N^{15} -labeled amino acids.

Ammonia (Table II) accounted for only a small fraction of the N^{15} found in the urine of non-acidotic rats injected intravenously with either ammonium glutamate or glutamine. The ratio, N^{15} concentration in urinary ammonia to N^{15} concentration in urinary urea, was greater following the intravenous administration of glutamine or ammonium glutamate to rats than following the oral administration of ammonium citrate (25) or amino acids (24, 26-29). This was probably due in part to the very high N^{15} concentration in the ammonia excreted during the 1st hour following

TABLE III

*Ratio of N^{15} in Liver to Total N^{15} in Body of Rats Following Intravenous Administration of $N^{15}H_4$ Glutamate and Glutamine Containing N^{15} in Amide Group**

Time	Per cent of administered N^{15} in body†		Per cent of administered N^{15} in liver		N^{15} in liver N^{15} in body	
	Glutamine	NH_4 glutamate	Glutamine	NH_4 glutamate	Glutamine	NH_4 glutamate
15 min.	100	100	13.5	10.7	0.14	0.11
1 hr.	87.5	90.3	9.2	10.1	0.11	0.11
6 hrs.	61.9	49.9	5.9	4.5	0.10	0.09
24 "	34.0	33.9	5.1	4.6	0.15	0.14

* Each value represents the analysis of a pooled sample from four rats receiving a total of 19 mg. of amide nitrogen (14.3 atom per cent excess of N^{15}) in the experiments with glutamine and 20 mg. of ammonia nitrogen (14.6 atom per cent excess of N^{15}) in the experiments with NH_4 glutamate.

† This value was obtained by subtracting the percentage of N^{15} recovered in the urine from 100.

injection, when the ratio was 4.45 for glutamine and 6.0 for ammonium glutamate. 24 hours after injection, however, the ratio had fallen to 1.33 for glutamine and 3.35 for ammonium glutamate. The difficulty in comparing the results obtained here with those following oral administration of labeled ammonium citrate or amino acids is that in the latter type of experiments the N^{15} was administered, as a constituent of the diet, over a period of 3 days, while in the present investigation a single dose of a much smaller amount of N^{15} was injected.

SUMMARY

1. The intravenous injection of isotopic ammonium glutamate or glutamine to rats was followed by a rapid removal of the isotope from the blood and relatively slight accumulation of isotope in the liver or kidneys.

2. Intravenously administered isotopic ammonium glutamate and the amide nitrogen of intravenously administered isotopic glutamine were largely converted to urea and excreted in the urine. With either of these substances only a small fraction of the injected isotope was excreted as ammonia.

BIBLIOGRAPHY

1. Friedberg, F., and Greenberg, D. M., *J. Biol. Chem.*, **168**, 411 (1947).
2. Awapara, J., and Marvin, H. N., *J. Biol. Chem.*, **178**, 691 (1949).
3. Foster, G. L., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **127**, 319 (1939).
4. Rittenberg, D., Schoenheimer, R., and Keston, A. S., *J. Biol. Chem.*, **128**, 603 (1939).
5. Sprinson, D., A symposium on the use of isotopes in biology and medicine, Madison, 182 (1948). Sprinson, D. B., and Rittenberg, D., *J. Biol. Chem.*, **180**, 707 (1949).
6. Archibald, R. M., *Chem. Rev.*, **37**, 161 (1945).
7. Krebs, H. A., *Biochem. J.*, **29**, 1951 (1935).
8. Speck, J. F., *J. Biol. Chem.*, **168**, 403 (1947).
9. Greenstein, J. P., and Leuthardt, F., *Arch. Biochem.*, **15**, 449 (1947).
10. Errera, M., and Greenstein, J. P., *J. Biol. Chem.*, **178**, 495 (1949).
11. Ferdman, D. L., Frenkel, S. R., and Silakova, L. I., *Biokhimiya*, **7**, 43 (1942).
12. Hamilton, P., *J. Biol. Chem.*, **145**, 711 (1942).
13. Archibald, R. M., *J. Biol. Chem.*, **154**, 643 (1944).
14. Archibald, R. M., *J. Biol. Chem.*, **154**, 657 (1944).
15. Cohen, P. P., *Biochem. J.*, **33**, 551 (1939).
16. Van Slyke, D. D., Phillips, R. A., Hamilton, P. B., Archibald, R. M., Futcher, P. H., and Hiller, A., *J. Biol. Chem.*, **150**, 481 (1943).
17. Leuthardt, F., *Z. physiol. Chem.*, **252**, 238 (1938).
18. Bach, S. J., *Biochem. J.*, **33**, 1833 (1939).
19. Krebs, H. A., *Biochem. J.*, **36**, 758 (1942).
20. Bernheim, F., and Bernheim, M. L. C., *J. Biol. Chem.*, **178**, 903 (1949).
21. Leuthardt, F., and Glasson, B., *Helv. chim. acta*, **29**, 1344 (1946).
22. Bergmann, M., Zervas, L., and Salzmann, L., *Ber. chem. Ges.*, **66**, 1288 (1933).
23. Fruton, J. S., *J. Biol. Chem.*, **165**, 333 (1946).
24. Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **153**, 401 (1944).
25. Schoenheimer, R., The dynamic state of body constituents, Cambridge, 51 (1942).
26. Schoenheimer, R., Ratner, S., and Rittenberg, D., *J. Biol. Chem.*, **130**, 703 (1939).
27. Weissman, N., and Schoenheimer, R., *J. Biol. Chem.*, **140**, 779 (1941).
28. Ratner, S., Rittenberg, D., Keston, A. S., and Schoenheimer, R., *J. Biol. Chem.*, **134**, 665 (1940).
29. Wu, H., and Rittenberg, D., *J. Biol. Chem.*, **179**, 847 (1949).

METABOLISM OF *l*-ASCORBIC ACID AND *L*-TYROSINE IN GUINEA PIG LIVER

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The experiments of Sealock *et al.* (1-3) and of Levine *et al.* (4) have given reasons for believing that one of the functions of ascorbic acid in the animal body is to facilitate the metabolism of *L*-tyrosine and *L*-phenylalanine.

Sealock and Silberstein (2) showed that the administration of tyrosine orally to scorbutic guinea pigs resulted in the excretion of metabolites containing the benzene ring, accounting for up to 80 per cent of the original tyrosine. Abnormal metabolites were not excreted by animals receiving *l*-ascorbic acid. Levine *et al.* (4) obtained similar results with premature infants.

Lan and Sealock (5) examined the livers of guinea pigs, both control and scorbutic, for ability to oxidize tyrosine as measured by increased Q_{O_2} in slices. The livers from scorbutic animals showed diminished ability to oxidize the tyrosine when compared with livers from guinea pigs receiving adequate ascorbic acid. The oxidizing ability of scorbutic liver slices could be restored by the addition of ascorbic acid.

Darby *et al.* (6) could find no difference in rate of metabolism of *L*-tyrosine or conjugation of phenol (as measured by disappearance of the hydroxy-phenyl groups) in liver slices from scorbutic and non-scorbutic guinea pigs. They concluded that the main defect in the metabolism of aromatic compounds by scorbutic guinea pigs is the inability of the scorbutic liver to oxidize the side chain of tyrosine rather than to oxidize the ring or conjugate the phenolic group.

However, Painter and Zilva (7) pointed out that excessive doses of tyrosine were necessary for guinea pigs to excrete metabolites containing the benzene ring, and that this abnormal excretion could only be prevented by having the guinea pig tissues saturated with *l*-ascorbic acid. They concluded that there was not necessarily a connection between the normal functions of *l*-ascorbic acid and normal metabolism of *L*-tyrosine.

The experiments reported below support the view that tyrosine metabolism in the liver is dependent on the presence of *l*-ascorbic acid.

Methods

Animals and Diets—Guinea pigs weighing between 200 and 250 gm. were fed, *ad libitum*, the scorbutigenic diet recommended by Dawbarn (8).

The control guinea pigs were given a daily oral supplement of 5.0 mg. of ascorbic acid. The animals receiving the scorbutogenic diet developed signs of acute scurvy in 15 to 20 days. An animal was not regarded as scorbutic until it had lost considerable weight and at autopsy showed considerable hemorrhage about the thighs.

Preparation of Liver Slices, Homogenates, and Supernatants—The animals were killed by a blow on the head and the livers removed after exsanguination.

The slices were made according to Cohen's description of Deutsch's method (9).

The homogenates were prepared by macerating the whole liver in 4 to 5 times its weight of 1 per cent K_2HPO_4 (pH 7.4) for 90 seconds in a Waring blender. After straining through muslin, the pH was readjusted to 7.4.

The homogenate, if centrifuged for 5 minutes at approximately $3000 \times g$, separated into a sediment with no activity towards tyrosine and a fluid with tyrosine-oxidizing properties, which is referred to below as the "supernatant." The supernatant preparation was much easier to pipette than whole liver homogenates.

Manometry—The oxygen consumption was measured in Warburg flasks of approximately 18 ml. capacity at 37° . 0.2 ml. of 20 per cent KOH plus a filter paper wick was used in the center well. After an equilibration period of 15 minutes, the taps were closed, the initial reading taken, and the substrate tipped in.

Chemical—Tyrosine was determined after the manometric incubation period by Lugg's method (10). Metaphosphoric acid (5 per cent) was used to precipitate the residual protein in the solutions from which slices were removed and in the homogenate preparations. The color developed was determined by means of a photoelectric absorptiometer. This method was found to give good reproducibility and only monohydroxyphenyl compounds were measured. Practically no blanks were found with the various tissue preparations to which no tyrosine had been added.

Nitrogen was determined by a Kjeldahl digestion (2 ml. of liver homogenate, 20 ml. of concentrated H_2SO_4 , 20 gm. of K_2SO_4), the ammonia being distilled in a Markham still (11) into 1 per cent boric acid.

Results

Slices—The oxygen consumption and tyrosine metabolism in the control and scorbutic guinea pig liver slices are shown in Table I. These results show a considerable difference in the rate of disappearance of tyrosine in control and scorbutic liver slices. Also the addition of 0.3 mg. of *l*-ascorbic acid restores in part the ability by the scorbutic livers to metabolize tyrosine.

Homogenates—Figures for increased oxygen consumption due to added tyrosine, and for tyrosine metabolism in homogenates prepared from control and scorbutic livers, are given in Table II.

TABLE I

Oxygen Consumption and Tyrosine Metabolism in Control and Scorbutic Liver Slices

Approximately 100 mg. (wet weight) of liver slices in 2 ml. of Krebs-Ringer phosphate and 2.3 mg. of tyrosine suspended in 0.5 ml. of Ringer's solution. Gas phase oxygen. The figures in parentheses indicate the number of guinea pigs in the group.

Condition of liver	Addition to reaction flask	Increased Q_{O_2} due to addition of tyrosine	Tyrosine metabolism disappearing per hr. per mg. of tissue, dry weight
			μM
Control	None	2.83 (3)	0.0382 (5)
Scorbutic	"	0.49 (3)	0.0125 (4)
"	0.3 mg. l-ascorbic acid	2.40 (2)	0.0288 (4)

TABLE II

Effect of Addition of Tyrosine on Oxygen Consumption and Tyrosine Metabolized in 90 Minutes per Mg. of Nitrogen of Liver Homogenates

2 ml. of homogenate in each flask; 0.5 mg. of tyrosine added in 0.5 ml. of buffer. Gas phase air.

	Control animals		Scorbutic animals	
	Increased oxygen consumption due to tyrosine	Tyrosine metabolized	Increased oxygen consumption due to tyrosine	Tyrosine metabolized
	$\mu l.$	μM	$\mu l.$	μM
	7.71	0.177	5.35	0.199
	7.95	0.180	5.20	0.125
	5.29	0.118	5.15	0.118
	5.15	0.114	7.30	0.160
	6.80	0.150	6.50	0.145
Mean	6.58	0.148	5.90	0.131
Molecular ratio, O_2 to tyrosine	1.98		2.01	

From these figures it is seen that there is a slight but not significant decrease in tyrosine-oxidizing capacity in scorbutic liver homogenates compared with those of the control animals. In both the control and scorbutic homogenates, 2 molecules of oxygen were consumed for each molecule of tyrosine metabolized.

Supernatants—Table III gives data showing the activity towards tyrosine of supernatant solutions with and without added *l*-ascorbic acid from the control and scorbutic animals. It is seen that *l*-ascorbic acid stimulated the disappearance of tyrosine from the supernatant solutions, the stimulation being significant only in the case of the scorbutic preparations. The oxygen consumption due to added tyrosine ran parallel with the tyrosine disappearance in all cases, maintaining a ratio of 2 molecules of oxygen for each molecule of tyrosine.

Specificity of l-Ascorbic Acid in Tyrosine-Oxidizing System—Isoascorbic acid and *d*-glucoascorbic acid, substances similar in structure to *l*-ascorbic acid, were tested for their ability to stimulate tyrosine disappearance in the supernatant preparation. Neither exhibited any effect whatever on the tyrosine-oxidizing system, even in quantities up to 2 mg. compared to 0.3

TABLE III

Effect of l-Ascorbic Acid on Metabolism of Tyrosine in Supernatant Fraction from Livers of Control and Scorbutic Guinea Pigs

2 ml. of supernatant in each flask with 0.5 mg. of L-tyrosine in 0.5 ml. of buffer. Gas phase air. Duration of manometric run, 90 minutes. Four experiments in each group.

Condition of liver	Additions	Tyrosine metabolized μM	Significance of difference
Control	None	1.20	Not significant, $P > 0.05$
	0.3 mg. ascorbic acid	1.31	
Scorbutic	None	1.18	Significant, $0.02 > P > 0.01$
	0.3 mg. ascorbic acid	1.65	

mg. of *l*-ascorbic acid (*d*-glucoascorbic acid is generally considered to have one-twentieth the antiscorbutic activity of *l*-ascorbic acid). The 0.3 mg. of *l*-ascorbic acid added to the same preparation increased the disappearance of tyrosine. It has been reported that the behavior of folic acid administered orally (12), or added to liver tissue *in vitro* (13), is similar to that of *l*-ascorbic acid. With the supernatant preparation, 0.1 mg. of folic acid stimulated tyrosine disappearance in the same manner as did *l*-ascorbic acid.

DISCUSSION

These results together with those of Lan and Sealock (5) indicate that *l*-ascorbic acid plays a part in the metabolism of tyrosine in the liver as judged by oxygen consumption and disappearance of hydroxyphenyl groups. However, the results do not support the contention of Darby *et al.* (6) that the main defect in the metabolism of aromatic compounds in scorbutic

guinea pigs is the inability to oxidize the side chain of tyrosine rather than the inability to oxidize the ring or conjugate the phenolic group. Indeed, it is hard to reconcile this view with the experiments of Sealock *et al.* (1-3) *in vivo*, showing the excretion of metabolites containing most of the aromatic ring which has been consumed as tyrosine, but which, however, contained side chains in various but incomplete stages of oxidation.

At present it does not seem possible to give an explanation for the different behavior of liver slices and homogenates.

The ratio of 2 molecules of oxygen consumed for each molecule of tyrosine disappearing has been observed before by various investigators. In the results above, it is seen that the addition of *l*-ascorbic acid does not alter this ratio. This suggests that the addition of *l*-ascorbic acid does not alter the type of oxidation. This does not support the contention of Painter and Zilva (7) that *l*-ascorbic acid is functioning as an adaptation of the animal to high L-tyrosine intakes.

l-Ascorbic acid is probably the only member of the structurally related ascorbic acid family that will stimulate tyrosine disappearance. However, added folic acid will give the same effect as *l*-ascorbic acid, both in the whole animal (12) and in liver tissue (13). Also in folic acid deficiency, the tyrosine metabolism seems to be deranged in the liver, as in scurvy (14). These facts all indicate that both *l*-ascorbic acid and folic acid are essential components of the L-tyrosine-metabolizing system.

SUMMARY

1. The oxidation of tyrosine in slices of guinea pig liver was followed by measurement of oxygen consumption and determination of the disappearance of hydroxyphenyl groups. Both methods showed that oxidation of tyrosine in the liver slice depended upon the *l*-ascorbic acid nutrition of the animal, and in the case of liver slices from scorbutic animals the oxidation could be restored to non-scorbutic values by addition of *l*-ascorbic acid.

2. By use of similar techniques no difference could be seen in rate of metabolism of tyrosine in whole liver homogenates from the control and scorbutic guinea pigs. However, addition of *l*-ascorbic acid stimulated tyrosine oxidation in supernatant preparations from liver homogenates.

3. *l*-Ascorbic acid seems specific in the ascorbic acid family in this effect, as isoascorbic acid and *d*-glucoascorbic acid had no effect whatever on the system. However, folic acid gave results similar to those with *l*-ascorbic acid.

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BIBLIOGRAPHY

1. Sealock, R. R., and Silberstein, H. E., *Science*, **90**, 517 (1939).
2. Sealock, R. R., and Silberstein, H. E., *J. Biol. Chem.*, **135**, 251 (1940).
3. Sealock, R. R., Perkinson, J. D., Jr., and Basinski, D. H., *J. Biol. Chem.*, **140**, 153 (1941).
4. Levine, S. Z., Gordon, H. H., and Marples, E., *J. Clin. Invest.*, **20**, 209 (1941).
5. Lan, T. H., and Sealock, R. R., *J. Biol. Chem.*, **155**, 483 (1944).
6. Darby, W. J., DeMeio, R. H., Bernheim, M. L. C., and Bernheim, F., *J. Biol. Chem.*, **158**, 67 (1945).
7. Painter, H. A., and Zilva, S. S., *Biochem. J.*, **41**, 511 (1947).
8. Dawbarn, M. C., *Australian J. Exp. Biol. and Med. Sc.*, **23**, 281 (1945).
9. Cohen, P. P., in Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric techniques and related methods for the study of tissue metabolism*, Minneapolis, 75 (1946).
10. Lugg, J. W. H., *Biochem. J.*, **31**, 1422 (1937).
11. Markham, R., *Biochem. J.*, **36**, 790 (1942).
12. Woodruff, C. W., and Darby, W. J., *J. Biol. Chem.*, **172**, 851 (1948).
13. Rodney, G., Swendseid, M. E., and Swanson, A. L., *Federation Proc.*, **6**, 419 (1947).
14. Rodney, G., Swendseid, M. E., and Swanson, A. L., *J. Biol. Chem.*, **168**, 395 (1947).

OXIDATION-REDUCTION POTENTIALS OF THE CYTOCHROME *c* SYSTEM*

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There have been many investigations of the oxidation-reduction potential of the cytochrome *c* system. Some of the results are concordant, though none at the time this work was undertaken included a complete potentiometric titration of the system over a range of pH values. Potentials have been reported for the system at varying pH values, usually within a narrow range bordering on the physiological. These results are not concordant, the most divergent being those reported by Green (10). The results of previous workers are summarized in Table I.

We report here complete potentiometric titrations, both oxidative and reductive, of the cytochrome *c* system at different pH and E'_0 values for the system throughout the pH range, 0.4 to 10.0. Since undertaking this work, a report of Paul (16) has appeared which covers the range of pH from 4 to 8.0 but with results and conclusions somewhat different from ours.

EXPERIMENTAL

Materials—Cytochrome *c* used in this study was prepared from fresh beef heart according to the method of Keilin and Hartree (14). Analysis for iron by the bipyridyl method revealed an iron content of 0.29 per cent. The preparation was dried from the frozen state and kept in a desiccator at 4°. Solutions were freshly prepared by dissolving the dry powder in the desired buffer solutions as needed. Concentrations of the cytochrome *c* solutions were determined spectrophotometrically at pH 7.2 after reduction with sodium hydrosulfite. Optical measurements were made with a Beckman spectrophotometer. The constants for cytochrome *c* as determined by Drabkin (9) were employed.

Constant hydrogen ion concentrations during the electrometric titrations were maintained by means of 0.1 M phosphate, acetate, borate, or glycine buffers. At the low pH values, solutions of sulfuric or hydrochloric acid were used. Final pH determinations of each buffer solution were made with a hydrogen electrode and a saturated HgCl half-cell at

* The data of this paper are taken from a dissertation submitted by F. L. Rodkey in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Harvard University.

30°. The potential of the saturated calomel electrode was determined by measuring the potential difference between this half-cell and the hydrogen electrode in the standard acetate, phosphate, HCl, and borate buffers suggested by Hitchcock and Taylor (11, 12), assuming the solutions to have the pH determined by these investigators.

Both oxidative and reductive titration curves of cytochrome *c* have been obtained. Potassium ferricyanide was used as an oxidant in the oxidative titrations. Both reduced phthiocol and chromous acetate have served as the reductant in the reductive titrations. Sodium hydrosulfite has not been used as a reducing agent because preliminary measurements indicated that this material was unsatisfactory. The titrating agents

TABLE I
Summary of Reported Oxidation-Reduction Potentials of Cytochrome c

Investigator and reference	Source of cytochrome <i>c</i>	Temperature	pH	<i>E'</i> °
		°C.		volt
Coolidge (7).....	Yeast		7.0	+0.260
			5.0	0.207
			4.5	0.235
Stone and Coulter (19).....	Bacteria		7.6	0.280
Green (10).....	Yeast	30	4.59	0.127
			7.14	0.123
			7.43	0.082
			8.88	0.082
			9.20	0.062
Ball (3).....	Beef heart	20	7.40	0.270
Wurmser and Fillitti-Wurmser (25)....	" "	25	5-8	0.253
Stotz <i>et al.</i> (20).....	" "	30	5-7.3	0.262
Paul (16).....	" "	20	7.0	0.260

were always freshly prepared under nitrogen in the desired buffer solution in order to minimize any change in pH. In most of the electrometric titrations, a low concentration of an electromotively active dye was added in order to accelerate attainment of equilibrium of the cytochrome *c* system with the electrodes. The dyes used as mediators were commercial samples and were used without further purification. Mediators were chosen whose oxidation-reduction potentials were as close as possible to potentials observed when the cytochrome *c* was 50 per cent reduced. The dye was always freshly dissolved in the desired buffer and added to the cytochrome *c* solution in such a manner that the final equivalent concentration of the dye was between 5 and 10 per cent of the molar concentration of cytochrome *c*. Titrations performed at a given pH, with or without a mediator present, gave titration curves which were similar through the range of

25 to 75 per cent reduction of the cytochrome *c*, though the titrations in the absence of a mediator reached equilibrium values more slowly.

Methods

Apparatus for the electrometric titration of cytochrome *c* is shown diagrammatically in Fig. 1. All titrations were carried out at 30°, temperature being maintained by a flow of water through the outer jacket of the titration vessel. For this purpose a constant temperature bath and

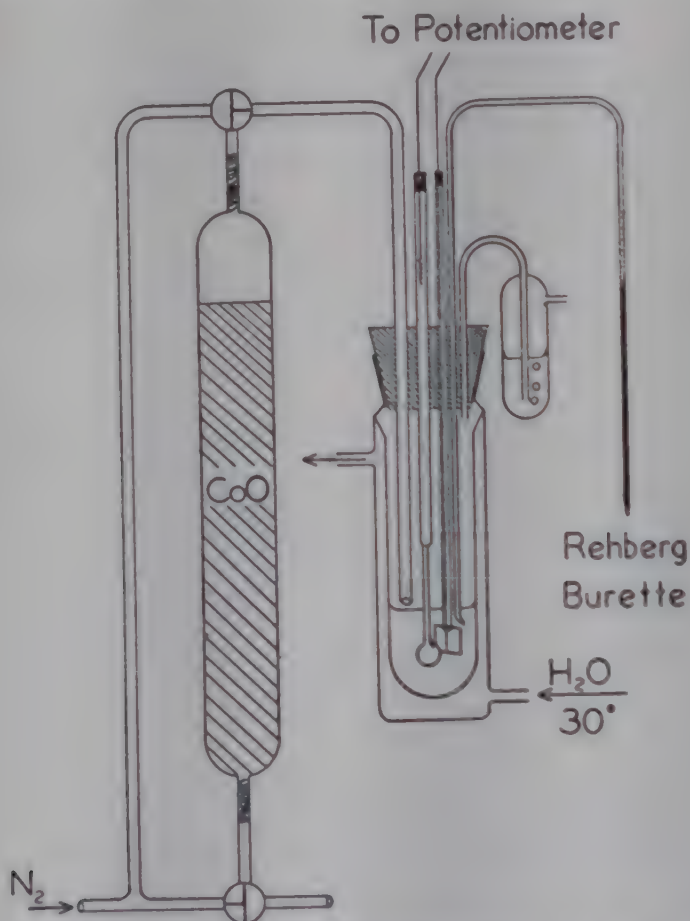


FIG. 1. Diagram of apparatus employed in electrometric titration of cytochrome *c*

circulating system manufactured by the Precision Scientific Company was employed. An atmosphere of nitrogen, freed of oxygen by passing over metallic copper at 600° and then through a glass tower of cobaltous oxide prepared according to the method of Pagel and Frank (15), was employed. In order to avoid liquid junctions, a bulb-type glass electrode served as the reference electrode. The potential of the reference electrode was checked before each run by measuring the potential established with quinhydrone in the buffer being used. In the alkaline region where quinhydrone was unstable, this method involved measuring the rate of change of the potential and then extrapolating back to the time quinhydrone was added to the buffer. Two gold-plated platinum electrodes were em-

ployed during each titration (one only is shown in Fig. 1). Titrating agents were added from a Rehberg micro burette whose total capacity was 250 c.mm. The inner portion of the titration vessel was 30 mm. in diameter, allowing titration and potential measurements to be performed on 5 or 10 ml. of cytochrome *c* solution.

The whole apparatus as shown in Fig. 1 was mounted on a board hinged at the bottom. Stirring of the solution contained in the titration vessel was accomplished by a gentle to and fro motion of the board. Potentials

TABLE II
Reductive Titration of Cytochrome c

pH 6.77 (phosphate); mediator, *o*-chlorophenol indophenol (2.5×10^{-6} M) temperature 30°; titrating agent, reduced phthiocol (about 0.015 N); cytochrome *c* concentration, 1×10^{-4} M; $d = 2.33$ c.mm.

Amount of titrating agent added		Reduction	E_h (observed)	E_o^\dagger (calculated), $n = 1$	Deviation from average
Total y	Corrected $y - d^*$				
c.mm.	c.mm.	per cent	volt	volt	volt
10	7.67	11.25	+0.2918	+0.2479†	
20	17.67	25.92	0.2863	0.2589	+0.0028
30	27.67	40.59	0.2703	0.2613	+0.0052
40	37.67	55.26	0.2503	0.2558	-0.0003
50	47.67	69.93	0.2323	0.2543	-0.0018
55	52.67	77.70	0.2213	0.2539	-0.0022
60	57.67	84.60	0.2093	0.2539	-0.0022
65	62.67	91.95	0.1923	0.2547	-0.0014
Average.....				0.2561	

* See Clark and Perkins (5) for the meaning of this term.

† No corrections have been made to the potential readings for the presence of the mediator, since, in the absence of information on the purity of the commercial dyes employed, no accurate corrections are possible. We have calculated, however, that the maximum error to be encountered by our failure to make such corrections is of the order of 2.5 millivolts.

‡ Not included in the average.

were measured with a Leeds and Northrup No. 7660 amplifying potentiometer, the necessary shielding precautions being observed. The suitability of this apparatus was first established by titrating several electromotively active dyes whose potentials were known.

Electrometric titration curves were performed on 10 ml. of cytochrome *c* solution, approximately 1×10^{-4} M. Equilibrium potentials (constant values for three consecutive readings at 15 minute intervals) were observed after addition of each increment of titrating agent in the usual fashion. Both graphical and mathematical analyses of the data obtained

were employed to establish the potential at 50 per cent reduction of the cytochrome *c*. The oxidation-reduction potential was also determined at several pH values by the method of mixtures. In this method the equilibrium potential was measured in a solution containing known amounts of both reduced and oxidized cytochrome *c*. From these data the potential at 50 per cent reduction of the cytochrome *c* was calculated.

TABLE III

Reductive Titration of Cytochrome c

pH 1.20 (HCl); mediator, indigotetrasulfonate (3.0×10^{-6} M); temperature 30° ; titrating agent, reduced phthiocol (about 0.015 N); cytochrome *c* concentration 1×10^{-4} M; $d = 11.0$ c.mm.

Amount of titrating agent added		Reduction	E_h (observed)	E'_0 (calculated), $n = 1$	Deviation from average
Total y	Corrected $y - d$				
c.mm.	c.mm.	per cent	volt	volt	volt
15	4.0	5.71	+0.3814	+0.3082	-0.0028
20	9.0	12.83	0.3614	0.3114	+0.0004
25	14.0	20.00	0.3459	0.3097	-0.0013
30	19.0	27.15	0.3359	0.3104	-0.0006
35	24.0	34.28	0.3264	0.3096	-0.0014
40	29.0	41.50	0.3194	0.3104	-0.0006
45	34.0	48.60	0.3144	0.3130	-0.0020
50	39.0	55.80	0.3084	0.3145	+0.0035
55	44.0	62.80	0.2989	0.3126	+0.0016
60	49.0	70.00	0.2894	0.3115	+0.0005
65	54.0	77.20	0.2739	0.3176	+0.0066
70	59.0	84.30	0.2634	0.3061	-0.0049
Average				+0.3112	

Results

In Table II the results of titrating 10 ml. of 1×10^{-4} M oxidized cytochrome *c* at pH 6.77 with reduced phthiocol are shown. The mediator was *o*-chlorophenol indophenol. Stable potentials were obtained very slowly (3 to 4 hours) outside the range of 25 and 75 per cent reduction. Through the middle portion of the curve, however, a stable potential was observed within 30 to 60 minutes after addition of the reducing agent. All potentials are referred to the potential of the standard normal hydrogen electrode in the usual manner. Results of titrating 10 ml. of 1×10^{-4} M oxidized cytochrome *c* with reduced phthiocol at pH 1.20 are shown in Table III. Indigotetrasulfonate was employed as mediator in this titration.

The observed data for these two titration curves were treated by the

method of Reed and Berkson (18) in the manner first used by Clark and Perkins (5). These results are shown in Tables II and III. The potentials determined correspond very well to those predicted from the theoretical equation

$$E_h = E'_0 + \frac{0.0601}{n} \log \frac{[\text{Oxidant}]}{[\text{Reductant}]} \quad (1)$$

TABLE IV
*E'*₀ Values of Cytochrome *c*

pH	Buffer	Method	Titration agent	Mediator*	<i>E'</i> ₀ volt
0.40	HCl	Reductive titration	Reduced phthiocol	1	+0.4119
0.81	"	Oxidative "	K ₃ Fe(CN) ₆	1	0.3582
0.90	H ₂ SO ₄	Reductive "	Chromous acetate	2	0.3853
1.20	HCl	" "	Reduced phthiocol	1	0.3112
1.76	Phosphate	Oxidative "	K ₃ Fe(CN) ₆	1	0.2548
1.76	"	Reductive "	Reduced phthiocol	None	0.2523
2.12	"	" "	" "	"	0.2404
2.56	"	" "	" "	"	0.2613
4.20	Acetate	Mixtures		3	0.2508
5.13	"	"		4	0.2509
6.23	Phosphate	Reductive titration	Reduced phthiocol	5	0.2522
6.23	"	" "	" "	5	0.2555
6.25	"	Mixtures		5	0.2452
6.25	"	"		5	0.2560
6.77	"	Reductive titration	Reduced phthiocol	5	0.2561
7.23	"	" "	" "	5	0.2560
7.91	"	Mixtures		5	0.2496
8.34	Borate	"		5	0.2175
8.93	"	"		6	0.1961
8.93	"	Reductive titration	Reduced phthiocol	6	0.1934
10.03	Glycine	Mixtures		5	0.1224
10.03	"	Reductive titration	Reduced phthiocol	5	0.1066

* Mediator 1 indigotetrasulfonate, Mediator 2 indigodisulfonate, Mediator 3 methylene blue, Mediator 4 1-naphthol-2-sodium sulfonate 2,6-dichlorophenol, Mediator 5 *o*-chlorophenol indophenol and Mediator 6 Bindschedler's green.

where $n = 1$. These observations confirm earlier determinations of the oxidation-reduction equivalent of the cytochrome *c* system. At no pH investigated has there been any deviation from the theoretical potential values expected for a reversible oxidation-reduction system with $n = 1$.

Oxidation-reduction potentials determined for the cytochrome *c* system through the pH range 0.4 to 10.0 are given in Table IV. Values shown in Table IV include those determined both by electrometric titration

and by the method of mixtures. In some cases both oxidative and reductive titrations have been performed at the same pH value, showing no difference in the potentials observed when the cytochrome *c* system was 50 per cent reduced.

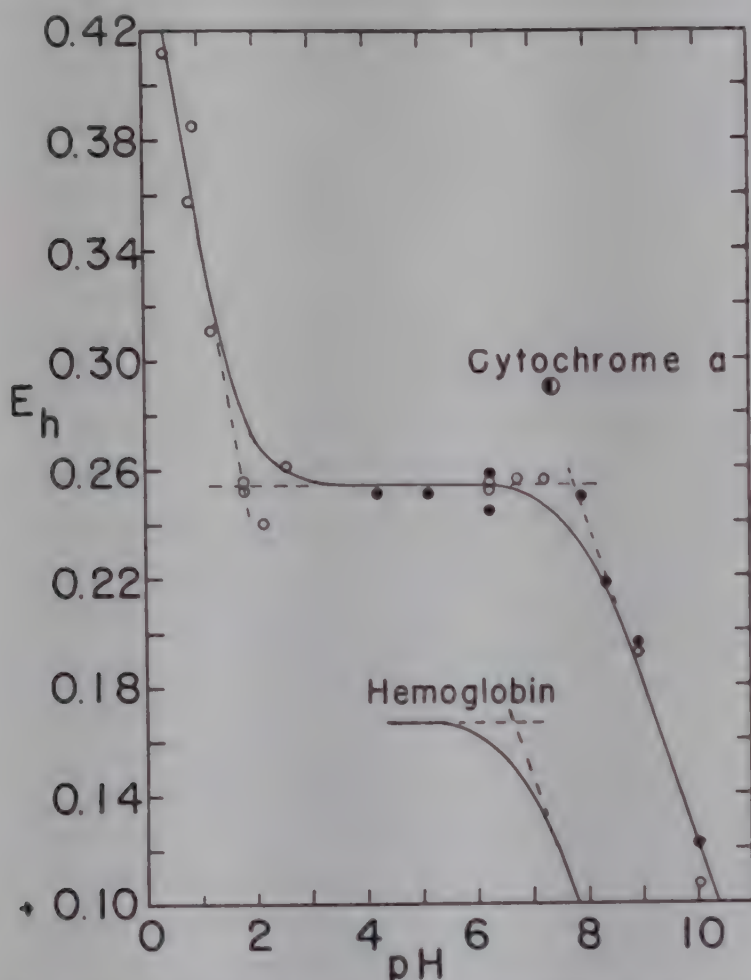


FIG. 2. E'_0 -pH curve of cytochrome *c*. O, represents the values determined by electrometric titration; ●, those determined by the method of mixtures. The values for the hemoglobin system are taken from Taylor and Hastings (21). The value for the cytochrome *a* system is taken from Ball (3). The curve for cytochrome *c* was drawn to fit values derived from the following equation:

$$E_h = E_0 - 0.06 \log \frac{[\text{Red.}]}{[\text{Ox.}]} + 0.06 \log (K_{r1}^2 + K_{r1}[H^+] + [H^+]^2) - 0.06 \log \frac{[H^+] + K_0}{[H^+]}$$

where $E_0 = 0.464$ volts, $K_{r1} = 1.78 \times 10^{-2}$, $K_0 = 1.58 \times 10^{-8}$, and $\frac{[\text{Red.}]}{[\text{Ox.}]} = 1$.

Values of the oxidation-reduction potential of cytochrome *c* throughout the pH range investigated are shown graphically by the E'_0 -pH curve in Fig. 2. For comparison with other heme proteins, the oxidation-reduction potentials of hemoglobin and cytochrome *a* are also included. The data given for hemoglobin are those of Taylor and Hastings (21), while the value for cytochrome *a* is that reported by Ball (3).

DISCUSSION

As may be seen from an inspection of Fig. 2, there are two changes in the slope of the experimentally determined E'_0 -pH curve within the pH range investigated. Between pH 1.75 and 7.8 the slope ($-\Delta E'_0/\Delta \text{pH}$) of this curve is 0.0 volt per pH. Since the titration curves clearly show that $n = 1$ for the cytochrome *c* system, this must mean that the oxidation or reduction of the pigment involves 1 electron and no hydrogen ions throughout this pH range. These results are in agreement with the earlier studies of Stotz, Sidwell, and Hogness (20) and of Wurmser and Fillitti-Wurmser (25).

In the neighborhood of pH 1.75 the E'_0 value of the system changes and becomes more positive with increasing acidity, at least as far as pH 0.40, the limit of our measurements. The change in potential observed between pH 1.75 and 0.40 corresponds closely to a value of 120 mv. per pH unit. A clean cut interpretation of these findings does not seem possible with the information at hand. For example, if we treat the data in the manner first outlined by Clark and Cohen (4) for a simple oxidation-reduction system, then we conclude that, acid to pH 1.75, the conversion of oxidized cytochrome *c* to the reductant involves the addition of 2 hydrogen ions. (That only 1 electron is still involved is indicated by the data in Table III.) The fact that the change in the E'_0 -pH curve is so abrupt at pH 1.75 would then further suggest that the dissociation constants of the two groups reacting with protons are nearly identical and that these two groups have similar chemical properties. Pursuing this interpretation, we are then faced with the question of the nature of the groups reacting. The involvement of two groups coordinated with the ferrous iron atom would appear to be the most logical explanation, since the valency state of the iron obviously affects their behavior. We are thus led to postulate that two amino acid residues of the protein moiety are displaced from their coordination with ferrous iron by their acceptance of a proton. Now if such an event does occur, then our data must also reflect the change in energy of association of the protein with the ferrous porphyrin group. We would then be dealing with the free energy changes of three distinct types of processes, the exchange of protons and electrons and of coordinating substance. However, our original interpretation of the 120 mv. slope of the E'_0 -pH curve was based upon reactions involving only the exchange of protons and electrons. We must therefore question the validity of this original interpretation and we are back where we began.

Nevertheless we are inclined to believe that our data do reflect some change occurring at acid pH values in the coordination pattern of reduced cytochrome *c*. The exact nature of the protein groups coordinated with the iron in cytochrome *c* is not known. If the break in the E'_0 -pH curve

at pH 1.75 is due to the addition of protons to these groups, then on the basis of known acid dissociation constants it would be reasonable to expect that carboxyl groups were involved. However, Theorell and Åkesson (24) have suggested that the imidazole groups of histidine residues are coordinated. If this is the case, then the free energy of coordination may be reflected in the displacement into this acid region of a dissociation normally occurring in a more neutral range. In this connection it may be recalled that Anson and Mirsky (1) were able to effect the release of heme from its coordination with globin by the treatment of hemoglobin with acid. A similar effect might thus be expected with cytochrome *c*, though the release of heme would not occur since Theorell (22) has indicated that a chemical bond exists between the protein moiety and the vinyl side chains of the porphyrin group.

We have attempted to obtain spectroscopic evidence for some change in the coordination state of iron in reduced cytochrome *c* in these acid regions without success. Also we may say that we have not been unmindful that in such acid environments unknown changes may occur in the structure of protein molecules. However, no evidence for such changes has been seen. Solutions of cytochrome *c* submitted to these acid environments show no change either in their absorption spectra or biological activity when returned to neutral solutions.

The careful studies of Clark and his coworkers (6) on a variety of hemochromogens indicate clearly that the complications of an iron porphyrin coordination system are many and the interpretation of oxidation-reduction potential data on such systems is best made in the light of supplementary data. The case of cytochrome *c* would appear to be no exception and we believe a more exact interpretation of our findings must await additional information. Magnetic susceptibility studies, for example, on reduced cytochrome *c* in this acid region might furnish valuable additional clues.

Theorell and Åkesson (24), on the basis of spectroscopic data, have presented evidence that a proton dissociates from oxidized cytochrome *c* with a pK of 2.50. We have observed no change in the slope of the E'_0 -pH curve through this region. It is possible that such a change is obscured by a simultaneous dissociation from the reductant. However, such an interpretation of the data is difficult to reconcile with the structure of cytochrome *c* at this pH which Theorell (23, 24) has set forth.

The other change in the slope of the E'_0 -pH curve occurs at pH 7.8, where it increases by 0.060 volt per pH. This change in slope may be interpreted as signifying that either a hydrogen ion dissociates from or a hydroxyl ion is added to oxidized cytochrome *c*, the pK value for the process being 7.8 in either case. The data given here do not permit a choice

between these two possibilities, but a comparison of the data with that available for the hemoglobin-methemoglobin system is instructive.

First let us consider the case in which the change in slope is to be ascribed to the dissociation of a hydrogen ion from the oxidant. In Fig 2, the data of Taylor and Hastings (21) for the hemoglobin-methemoglobin system are plotted. This system shows a change in slope similar to that for the cytochrome system; the pK value in this case, however, is 6.65. Now Coryell, Stitt, and Pauling (8) assign pK values of 5.3 and 6.65 to those two imino groups of the histidine residues of methemoglobin which are believed to be in the vicinity of the iron atom. The first pK value (5.3) is that of the imino group least favorably situated for coordination, and its pK value is believed to be unaltered by changes in the state of the valency of the iron atom. Thus no change in the oxidation-reduction potential of the system is to be expected from its dissociation. On the other hand, the group which shows a pK value of 6.65 for methemoglobin is altered by the valency state of the iron and it has a pK value of 7.81 in hemoglobin. Thus the change in the slope of the hemoglobin-methemoglobin system at pH 6.65 can most reasonably be attributed to the dissociation of a hydrogen ion from the oxidant of the system. By analogy, we might thus argue that a similar interpretation is valid for the cytochrome system. The higher pK value (7.8), in the case of the cytochrome system, would suggest that either a tighter coordination of an imino group from a histidine residue is being encountered or a chemically different group is involved.

If we accept this interpretation of the break in the E'_0 -pH curve of the cytochrome system, then again by analogy with the hemoglobin system we may expect the dissociation of a proton from reduced cytochrome *c* at a pH more alkaline than 7.8. In the hemoglobin system, a change in the valency of iron caused a difference of 1.16 units in the pK value of the oxidant and reductant. If the magnitude of this shift is similar for the cytochrome system, then the reductant of the system might be expected to have a group dissociating at $7.8 + 1.16$ or approximately 9.0. Paul (16), on the basis of spectroscopic observations, has assigned a pK value of 9.28 to some acid group dissociating in reduced cytochrome. Now it might be expected that as a result of this dissociation of a hydrogen ion from the reductant the E'_0 -pH curve of the cytochrome system would revert to a 0.0 slope in the region of pH 9.0. No such break in the potential curve has been observed by us. This does not necessarily mean, however, that the premise as outlined above is wrong. Our failure to observe any alteration in the slope of the E'_0 -pH curve in this pH region can be explained by the dissociation of a second group from the oxidant. Theorell and Åkesson (24) have presented evidence to indicate that the oxidant

of cytochrome *c* has a group dissociating at pH 9.35. Thus the effect on the potentials of the system by the dissociation of the first heme-linked group of reduced cytochrome would be counter-balanced by the dissociation of a second heme-linked group of oxidized cytochrome *c*.

We may now turn to the alternate interpretation that the break in the E'_0 -pH curve of the cytochrome system at pH 7.8 is due to the addition of a hydroxyl ion to the oxidant. Again support for this hypothesis may be obtained by comparison with methemoglobin. From the spectroscopic measurements of Austin and Drabkin (2) and the magnetic measurements of Coryell, Stitt, and Pauling (8), the pK value of 8.1 may be assigned to the formation of methemoglobin hydroxide. The addition of a hydroxyl ion to oxidized cytochrome *c* in this same pH region therefore does not seem unreasonable at first glance. It must, however, be remembered that all the evidence indicates that the groups coordinated with cytochrome iron are much more strongly linked than in the case of hemoglobin. The introduction of a hydroxyl group into ferric cytochrome might, therefore, be expected to occur at much higher pH values than in the case of methemoglobin. Such a supposition finds support in the observations of Potter (17) and Horecker and Kornberg (13) that cyanide reacts very slowly with oxidized cytochrome *c* in contrast to its behavior with methemoglobin. All in all, we are inclined to favor the interpretation that the break in the E'_0 -pH curve of the cytochrome *c* system at pH 7.8 is due to the dissociation of a hydrogen ion from the oxidant.

Paul (16) reported a change in the slope of the E'_0 -pH curve at pH 6.86. His data thus also indicate a single dissociation from the oxidant not matched by a similar dissociation from the reductant, but at a more acid pH than that observed by us. Though the potentiometric method for determination of dissociation constants of a compound like cytochrome *c* may be somewhat in error, it is difficult to understand why the pK values calculated from the two sets of data should disagree by a whole pH unit. At present this discrepancy cannot be explained. It is of interest to note that the few data of Paul in strongly alkaline solutions are very close to the E'_0 values reported in our study. The same is also true of the single determination which he performed in 0.1 N HCl.

SUMMARY

1. An apparatus for the measurement of the oxidation-reduction potentials of small quantities of biological materials is described and the results of its application to the cytochrome *c* system are presented.

2. Complete electrometric titration curves have been obtained throughout the pH range 0.4 to 10.0, and show that cytochrome *c* forms a reversible oxidation-reduction system involving a 1 electron change within this pH range.

3. The potential of the cytochrome *c* system remains constant at $+0.254$ volt throughout the pH range 1.75 to 7.8, signifying that no hydrogen ions are involved in the reaction within this pH range.

4. At pH values acid to 1.75, the potential of the system increases 120 millivolts for each unit decrease in pH value. This is interpreted to mean that the conversion of reduced cytochrome *c* to the oxidant involves not only the loss of 1 electron, but also the release of 2 hydrogen ions. It is suggested that these data may indicate a change in the coordination of the reduced iron atom with two groups of the protein moiety dissociating protons concurrently with pK values of 1.75.

5. In the pH region from 7.8 to 10.0, the potential of the system decreases 60 millivolts for each unit increase in pH value. The dissociation of a hydrogen ion or the addition of a hydroxyl ion to the oxidant with a pK value of 7.8 is indicated by these data.

BIBLIOGRAPHY

1. Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, **13**, 469 (1930).
2. Austin, J. H., and Drabkin, D. L., *J. Biol. Chem.*, **112**, 67 (1935-36).
3. Ball, E. G., *Biochem. Z.*, **295**, 262 (1938).
4. Clark, W. M., and Cohen, B., *Pub. Health Rep., U. S. P. H. S.*, **38**, 666 (1923).
5. Clark, W. M., and Perkins, M. E., *J. Am. Chem. Soc.*, **54**, 1228 (1932).
6. Clark, W. M., Davies, T. H., Porter, C. C., Taylor, J. F., Shack, J., Vestling, C. S., and Weisiger, J. R., *Federation Proc.*, **7**, 499 (1948).
7. Coolidge, T. B., *J. Biol. Chem.*, **98**, 755 (1932).
8. Coryell, C. D., Stitt, F., and Pauling, L., *J. Am. Chem. Soc.*, **59**, 633 (1937).
9. Drabkin, D. L., *J. Biol. Chem.*, **140**, 373 (1941).
10. Green, D. E., *Proc. Roy. Soc. London, Series B*, **114**, 423 (1934).
11. Hitchcock, D. I., and Taylor, A. C., *J. Am. Chem. Soc.*, **59**, 1812 (1937).
12. Hitchcock, D. I., and Taylor, A. C., *J. Am. Chem. Soc.*, **60**, 2710 (1938).
13. Horecker, B. L., and Kornberg, A., *J. Biol. Chem.*, **165**, 11 (1946).
14. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **122**, 298 (1937).
15. Pagel, H. A., and Frank, E. D., *J. Am. Chem. Soc.*, **63**, 1468 (1941).
16. Paul, K.-G., *Arch. Biochem.*, **12**, 441 (1947).
17. Potter, V. R., *J. Biol. Chem.*, **137**, 13 (1941).
18. Reed, L. J., and Berkson, J., *J. Phys. Chem.*, **33**, 760 (1929).
19. Stone, F. M., and Coulter, C. B., *J. Gen. Physiol.*, **15**, 629 (1932).
20. Stotz, E., Sidwell, A. E., Jr., and Hogness, T. R., *J. Biol. Chem.*, **124**, 11 (1938).
21. Taylor, J. F., and Hastings, A. B., *J. Biol. Chem.*, **131**, 649 (1939).
22. Theorell, H., *Biochem. Z.*, **298**, 242 (1938).
23. Theorell, H., *Advances in enzymology and related subjects*, New York, **7**, 265 (1947).
24. Theorell, H., and Åkesson, Å., *J. Am. Chem. Soc.*, **63**, 1812 (1941).
25. Wurmser, R., and Fillitti-Wurmser, S., *J. chim. phys.*, **35**, 81 (1938).

A METHOD FOR THE DETERMINATION OF FREE AMINO ACIDS IN RAT ORGANS AND TISSUES*

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In a previous study a microadaptation of the microbiological assay procedure was used to measure the concentration of free¹ amino acids in rat plasma (1). During the course of this investigation we became interested in the application of these methods to the analysis of free amino acids in other tissues of the rat. Studies on the concentration of free amino acids in the blood and other tissues might lead to a better understanding of protein and amino acid metabolism in the animal body. To our knowledge there have been no previous reports on the application of the microbiological assay procedure to the determination of amino acids in tungstic acid extracts of animal tissues. However, these extracts have been successfully employed by various workers (2-5) for determination of amino acid nitrogen and other non-protein constituents of animal tissues. The purpose of the present paper is to report the observations made in establishing methods for the microbiological determination of free amino acids in various tissues of the rat.

General Procedure

Adult male albino rats of the Sprague-Dawley strain were maintained on an 18 per cent casein-sucrose diet (1). After a fast of 9 to 12 hours the animals were anesthetized with approximately 10 mg. of sodium amytal per 100 gm. of body weight administered intraperitoneally in one dose. When plasma filtrates were studied, the blood was withdrawn and filtrates prepared as previously described (1). Each animal was then bled by decapitation.

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¹ Free as used throughout implies microbiologically available amino acids and may include certain combined forms as well as free amino acids.

Preparation of Tissue Extracts—The tissues chosen for study were the median lobe of the liver, the right posterior femoral muscles, the brain, and the spleen. Portions of tissues were rapidly excised, weighed on a sensitive torsion balance to the nearest 0.01 gm., and placed in 5 ml. of hot water in the homogenizing tubes. The tubes and samples were heated in a boiling water bath for 2 minutes, after which they were removed and cooled. The samples were homogenized in a Potter-Elvehjem glass homogenizer (6). After one complete passage of the homogenizing pestle through the samples, the required amounts of tungstic acid solution (Table I) were added and homogenization completed. The water required for dilution (Table I) was added and the tubes and contents allowed to stand at room temperature for 30 minutes with intermittent shaking. The precipitates were removed by centrifugation. The protein-free filtrates were neutralized to pH 7.0 with N NaOH and stored in the frozen state under toluene until analyzed.

Assay Procedure—The microbiologically available amino acids were determined as previously described (1, 7, 8). To facilitate large routine analyses for amino acids, a composite standard² was employed to establish the standard curves of the assay organisms. The Cannon dispenser and titrator³ were employed throughout.

The 5- to 10-fold dilutions of the fresh tissues (Table I) were suitable for determining the majority of amino acids. The few exceptions and the dilutions required were as follows: histidine in liver and spleen 20-fold, in muscle 30-fold; threonine in brain and muscle 10-fold; proline in muscle 10-fold, in spleen 20-fold. The calculation of results was based on the assumption that amino acids were equally distributed in the supernatant

² A dry mixture of crystalline amino acids of the following composite was prepared: 75 mg. of L-tryptophan, 169 mg. of L-histidine hydrochloride, 200 mg. of L-methionine, 250 mg. of L-proline, 250 mg. of L-tyrosine, 375 mg. of L-leucine, 400 mg. of DL-phenylalanine, 605 mg. of L-arginine hydrochloride, 750 mg. of DL-valine, 750 mg. of DL-isoleucine, 750 mg. of DL-threonine, and 1.372 gm. of hydrous L-lysine hydrochloride. This mixture was ground in a mortar and after drying was stored in a tightly stoppered bottle in a desiccator. Each month 0.5946 gm. of the dry mixture was dissolved in water with HCl and diluted to 100 ml. This concentrated composite solution was stored at 3° under toluene in a tightly stoppered bottle. Prior to each assay 4 ml. of the concentrated solution were neutralized and diluted to 100 ml. 1 ml. of this working composite standard provided the following quantities of L isomers of the amino acids: tryptophan 3 γ , histidine 5 γ , methionine 8 γ , proline 10 γ , tyrosine 10 γ , leucine 15 γ , phenylalanine 8 γ , arginine 20 γ , valine 15 γ , isoleucine 15 γ , threonine 15 γ , and lysine 40 γ . The composition of the dry composite remained constant throughout 1 year of use, and in all respects the growth responses of the assay microorganisms with the composite standard were identical with the responses obtained from individual amino acid standards.

³ Cannon, M. D., in press.

and the moisture remaining in the precipitate. Good agreement was found at the various assay levels over the entire range of the standard curves for all of the amino acids determined.

EXPERIMENTAL AND RESULTS

Treatment Prior to Precipitation—Since proteolytic enzymes are present in the animal tissues studied, it was necessary to destroy their action as rapidly as possible. This was accomplished by promptly boiling the samples. Data are presented in Table II to show the extent of proteolysis occurring in various periods of time in homogenates of boiled and unboiled samples. In this experiment the same tissues of several rats were pooled. By dividing each of the individual tissues into two parts, two comparable samples of each type of tissue were obtained; one was boiled and the other left untreated. The samples were homogenized, precipitated, and then

TABLE I
Quantities of Reagents Used in Extraction of 1 Gm. of Tissue

Sample	Protein-precipitating agent*	Water	Final dilution
	ml.	ml.	ml.
Liver.....	1.5	7.5	1:10
Brain.....	0.6	3.4	1:5
Muscle.....	1.2	2.8	1:5
Spleen.....	0.9	8.1	1:10

* The precipitating agent consists of 5 parts of 10 per cent sodium tungstate and 7 parts of 0.6 N sulfuric acid.

made to volume and allowed to stand at room temperature. At intervals of 10, 30, 120, and 360 minutes, portions of each homogenate were centrifuged and the resulting extracts neutralized and stored. The concentrations of three arbitrarily chosen amino acids, leucine, tryptophan, and threonine, were determined in each extract. In those instances in which no values are given, mechanical difficulties resulted in the loss of the samples.

The free amino acid concentrations in homogenates of unboiled liver, spleen, brain, and muscle were markedly increased by prolonged standing with the protein precipitants. On the other hand, amino acid concentrations of boiled sample extracts were not affected under the same conditions. This demonstrates conclusively that proteolysis can occur in a solution in contact with tungstic acid precipitate of tissue protein. It is an enzymatic change, since heat treatment arrested the proteolysis. Limited experiments have indicated that proteolysis occurred only if the precipitate was present. After the separation of extracts from the protein

precipitates of unboiled tissues, no increase was found in the extracts allowed to stand for long periods of time. However, 5-fold increases of microbiologically available amino acids were found in the respective precipitates standing for 6 hours at room temperature. Therefore, the tungstic acid completely precipitated the catheptic enzymes but did not destroy their activity.

TABLE II
Changes in Amino Acid Content of Precipitated Homogenates at Various Time Intervals

Sample	Time of standing	Leucine		Tryptophan		Threonine	
		Unboiled	Boiled	Unboiled	Boiled	Unboiled	Boiled
	min.	γ per gm.	γ per gm.	γ per gm.	γ per gm.	γ per gm.	γ per gm.
Liver	10	212	94	26.5	15.3	255	136
	30	361	90	41.5	14.2	293	125
	120	782	100	95.2	15.4	530	
	360	910	114	124	14.2	590	146
Spleen	10	1062	185	184	31.5	680	169
	30	1254	189	218	20.0	685	
	120	1404	212	308	30.6	996	175
	360	2608	186	450	20.5	1892	174
Brain	10	34.4		5.9		105	
	30	61.7	34.8	8.6	7.6	128	108
	120	112	30.8	17.1	7.7	161	109
	360	308	33.7	37.8	7.6	370	96.7
Muscle	10	59.4	33.0	11.0	6.9	123	133
	30	59.5	29.8	10.0	8.2	145	126
	120	87.0	34.6	12.7	8.0	138	125
	360	215	28.9	15.6	8.0	175	136
		γ per ml.	γ per ml.	γ per ml.	γ per ml.	γ per ml.	γ per ml.
Plasma	10	20.4		15.7		38.1	
	30	22.7	21.4	17.3	14.7	41.6	41.8
	360	23.1		15.7		43.1	

It may be noted in Table II that boiling the plasma prior to precipitation did not affect the values obtained. Therefore, it is reasonable to assume that no effect of boiling, other than the inactivation of catheptic enzymes, occurs. The observations of Christensen and Lynch (9) that tungstic acid extracts of blood plasma are not affected by prolonged contact with the protein precipitate are confirmed.

It is interesting to note that the rate of proteolysis varies with the tissues and the amino acid studied. A comparison of the amino acids in boiled and unboiled tissues indicates that spleen shows the largest proteolysis in the first 10 minute interval. The rate of proteolysis in the

spleen after 10 minutes, however, was not as great as that of the other tissues.

We have experimental evidence, not presented in this paper, that there is very little effect upon the free amino acid values obtained when there is only a short delay (less than 5 minutes) before boiling. A delay of 2 hours after boiling the tissue samples, but before precipitation of the proteins, did not affect the amino acid concentrations in the extract. When the excised tissues were immediately frozen by means of dry ice, no change of amino acid values was noted with delays up to 1 hour prior to boiling. These results indicate that it is advisable to freeze the organs and tissues promptly after excision if they cannot be boiled within 5 minutes. The advisability of prompt removal of tissues from the animal has been demonstrated by Luck (10).

TABLE III

Effect of Excess Precipitating Agent upon Free Amino Acid Content of Rat Tissues

Sample	Precipitating agents	Leucine	Tryptophan
	<i>ml. per gm.</i>	<i>γ per gm.</i>	<i>γ per gm.</i>
Liver	1.5 (Normal)	110	24.8
	1.8	101	19.3
	2.4	110	21.1
Brain	0.6 (Normal)	33.7	7.6
	0.9	39.1	7.7
	1.5	41.8	8.2
Muscle	1.2 (Normal)	50.4	11.0
	1.5	41.5	10.3
	2.1	45.8	11.4

The homogenizing procedure was adequate as described. No increase in amino acid values was obtained by longer periods of homogenization.

Tungstic Acid Precipitation—The procedure for precipitating the tissue proteins is a modification of that employed by Hier and Bergeim (11) for the precipitation of blood plasma proteins. To establish the best conditions for the precipitation of the proteins in other rat tissues, varied quantities of the precipitating agent were added to homogenates of each tissue. The presence of excess tungstate was measured by the addition of egg albumin and unprecipitated protein by heat coagulation or by the addition of more tungstate. The best proportions of tungstic acid to homogenates of the various tissues have been given earlier, in Table I.

Since excessive amounts of tungstate present in tissue extracts might inhibit the growth of the microorganisms employed for assaying the amino acids, extracts were prepared with more than the quantities of tungstic

acid ordinarily employed for the precipitation of the proteins. The results of this experiment for two representative amino acids are given in Table III. With the amounts of excess tungstate used there was no inhibition of the growth of the microorganisms. A further experiment indicated that the addition of 0.1 ml. of 10 per cent sodium tungstate to 1 ml. of an extract, prior to analysis, did not produce any change or drift in the values obtained at various assay levels.

Recovery of Amino Acids Added to Homogenates—To determine the reliability of the method of protein precipitation and the assay procedure itself, amino acid recovery experiments were performed. The amino acids

TABLE IV
*Recovery of Amino Acids Added to Homogenates Prior to Precipitation**

Amino acid	Liver	Brain	Muscle	Spleen
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Leucine	115	106	107	107
Phenylalanine	98	113	96	104
Tryptophan	99	98	108	103
Valine	106	111	107	109
Histidine	101	102	102	97
Lysine	101	95	92	87
Isoleucine	99	110	92	104
Proline	110	93	98	107
Tyrosine	102	115	106	97
Arginine	104	104	107	93
Threonine	100	90	114	106
Methionine	105	113	100	105
Mean	103	104	102	101

* These are the mean values of one to four recovery experiments.

to be recovered were added in solution to the various homogenates prior to protein precipitation, and the volume of water was reduced accordingly. One portion of each homogenate was precipitated without the addition of amino acids to provide values for the normal content of amino acids in 1 gm. of the tissues employed. Amino acids were added to aliquots of the homogenates that represented 1 gm. dilutions of the tissues. Prior to the separation of the recovery extracts from the precipitates by centrifugation, they were diluted with an equal volume of water to give suitable concentrations for the assays. Normal and recovery extracts were analyzed simultaneously for twelve amino acids. Recoveries were calculated after subtraction of the amino acids found on analysis of the tissues alone. Though this method of calculating the recoveries accentuates any error

that might be involved in the assay of the recovery extracts, acceptable recoveries were obtained with all tissues for the majority of amino acids determined (Table IV).

Hydrolysis of Protein-Free Extracts—To determine the relative quantities of bound⁴ and free amino acids present in protein-free extracts, portions of each of the extracts were analyzed before and after acid hydrolysis. Aliquots (20 ml.) of extracts of the various tissues were evaporated to dryness below 60° at 12 mm. pressure. The dried samples were hydrolyzed with 1.6 ml. of 3 N HCl for 5 hours at 15 pounds pressure. After hydrolysis the samples were neutralized and diluted to the original 20 ml.

TABLE V

Amino Acid Content of Tungstic Acid Extracts before and after Acid Hydrolysis

Amino acid	Plasma		Liver		Brain		Muscle		Spleen	
	Normal	Hydrolyzed	Normal	Hydrolyzed	Normal	Hydrolyzed	Normal	Hydrolyzed	Normal	Hydrolyzed
	γ per ml.	γ per ml.	γ per gm.	γ per gm.	γ per gm.	γ per gm.	γ per gm.	γ per gm.	γ per gm.	γ per gm.
Leucine	18.7	54.8	77.5	99.6	21.9	32.3	25.9	41.9	161	184
Phenylalanine . .	9.5	28.3	44.4	44.5	13.4	14.7	20.9	20.9	84.2	68.3
Tryptophan	16.4		15.0		6.1		6.9		27.7	
Valine	21.3	41.2	59.8	64.2	11.9	22.0	32.2	30.9	119	135
Histidine	5.8	12.3	55.9	61.4	8.7	19.1	62.5	318	67.4	62.1
Lysine	34.7	56.3	82.4	87.9	27.9	29.3	92.1	92.1	142	201
Isoleucine	9.7	18.5	34.7	32.2	7.1	16.3	29.7	30.9	96.2	124
Proline	26.6	71.7	86.5	98.7	16.6	30.1	70.6	74.0	142	139
Tyrosine	18.3	31.1	47.0	41.5	20.6	18.0	43.0	38.1	85.9	86.6
Arginine	25.6	36.0	7.4	5.0	18.8	16.8	63.0	45.1	61.6	71.1
Threonine	43.2	79.6	113	124	125	111	145	140	130	208
Methionine	9.1	17.2	35.4	30.4	13.5	10.7	21.8	17.8	48.7	59.0

volumes. The amino acid concentrations in the extracts before and after hydrolysis were determined simultaneously. The results are shown in Table V.

The increase in microbiologically available amino acids after hydrolysis was greatest in the plasma extracts. The hydrolyzed extracts of liver, brain, muscle, and spleen showed only a few large increases in available amino acid concentrations. The leucine concentrations of all tissue extracts were increased by hydrolysis. Other significant increases occurred in the concentrations of lysine and threonine in spleen; isoleucine, proline, and histidine in brain; and histidine in muscle extracts. The outstanding increase of histidine in muscle extracts is probably a result of the hydroly-

⁴ As used here, bound means microbiologically unavailable forms of the amino acid which become available after acid hydrolysis.

sis of carnosine, since this peptide is found in appreciable amounts in muscle tissue (12).

The concentrations of several amino acids in each tissue extract, except plasma, were decreased upon hydrolysis. We can only assume that these decreases were a result of destruction of free amino acids by the acid hydrolysis or a result of the peptides being more active than amino acids for the microorganism. The concentrations of tryptophan in hydrolyzed extracts were not determined, as this amino acid is known to be destroyed by acid hydrolysis.

This experiment indicates that tungstic acid extracts from blood plasma contain greater quantities of combined amino acids than those from liver, brain, muscle, and spleen. These observations may be of some importance, since some peptides can replace one or more of their constituent amino acids for lactic acid bacteria (13-15).

Other Considerations—Hier and Bergeim (11) report that amino acid concentrations in tungstic acid extracts of blood plasma were not affected by prolonged storage at low temperatures. Our observations upon repeated assays of stored tissue extracts are in agreement with their findings. Assay of extracts before and after storage for 90 days or more gave the same values, within the accuracy of the assay methods.

The effect of anesthesia of the animals upon tissue amino acid levels has not been investigated thoroughly. However, limited experiments with plasma amino acids indicated that the values are not significantly affected by the anesthetic. These results confirm those of Hier and Bergeim (11).

In subsequent communications applications of these procedures to animals under various experimental conditions will be reported. Thus, more conclusive evaluations of these methods will be obtained.

SUMMARY

1. Methods for preparing tungstic acid extracts of liver, brain, muscle, and spleen suitable for analysis of free amino acids have been described.
2. A microadaptation of the microbiological procedure has been applied to the analysis of the apparently free amino acids in these extracts. The various steps in the procedure have been critically examined.

BIBLIOGRAPHY

1. Henderson, L. M., Schurr, P. E., and Elvehjem, C. A., *J. Biol. Chem.*, **177**, 815 (1949).
2. Hammett, F. S., *J. Biol. Chem.*, **48**, 127 (1921).
3. Denis, W., *J. Biol. Chem.*, **54**, 693 (1922).
4. Kiech, V. C., and Luck, J. M., *J. Biol. Chem.*, **77**, 723 (1928).
5. Ayres, G. B., and Lee, M., *J. Biol. Chem.*, **115**, 139 (1936).

6. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
7. Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, **172**, 15 (1948).
8. Henderson, L. M., Brickson, W. L., and Snell, E. E., *J. Biol. Chem.*, **172**, 31 (1948).
9. Christensen, H. N., and Lynch, E. L., *J. Biol. Chem.*, **166**, 87 (1946).
10. Luck, J. M., *J. Biol. Chem.*, **77**, 1 (1928).
11. Hier, S. W., and Bergeim, O., *J. Biol. Chem.*, **161**, 717 (1945).
12. von Fürth, O., and Schwarz, C., *Biochem Z.*, **30**, 413 (1911).
13. Hegsted, D. M., *J. Biol. Chem.*, **157**, 741 (1945).
14. Agren, G., *Acta physiol. Scand.*, **13**, 347 (1947).
15. Krehl, W. A., and Fruton, J. S., *J. Biol. Chem.*, **173**, 479 (1948).

THE DETERMINATION OF FREE AMINO ACIDS IN RAT TISSUES*

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In a previous paper (1) methods for the preparation of protein-free extracts of rat tissues were reported. These extracts were found to be suitable for the estimation of free¹ amino acids by the microbiological assay procedure. In the present paper we have employed these methods to determine the characteristic concentrations of free amino acids in several tissues of adult, male albino rats.

EXPERIMENTAL

Care of Animals—The adult rats were maintained on a stock diet for 2 weeks and then given a purified ration at least 2 weeks prior to the beginning of the studies. The purified ration had the following composition: sucrose 71, crude casein 18, Salts IV (2) 4, corn oil 5, and a dry vitamin mixture (3) 2 per cent.

Three groups of animals were employed: Group I, five male rats of the Sprague-Dawley strain weighing from 300 to 350 gm., received the ration *ad libitum* until sacrificed. Group II, nine Sprague-Dawley male rats from 350 to 400 gm. in weight, were fasted 12 hours before sacrifice. Group III, eight Holtzman male rats from 200 to 250 gm. in weight, were fasted for 12 hours before sacrifice.

Procedure—The animals were anesthetized with sodium amytal, blood was withdrawn from the heart, and plasma extracts were prepared as described by Henderson *et al.* (3). The rats were bled by decapitation: and the median lobe of the liver, the right posterior femoral muscles, the brain, and the spleen of the animals were excised immediately. Tungstic acid extracts of these tissues were prepared as previously described (1).

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¹ Free as used throughout implies microbiologically available amino acids and may include combined forms as well as free amino acids.

except that the tissues of the animals of Group III were frozen in dry ice immediately after removal from the animal body. The extracts were stored in stoppered test-tubes under toluene at -5° until analyzed.

The amino acid assay procedures have been previously described (4). Since only small quantities of tissue extracts were obtained from individual rats, duplicate levels of only 0.04, 0.06, and 0.08 ml. of each extract were assayed for amino acids. It had been previously observed that no significant drift of values occurred when 0.02 to 0.1 ml. levels were employed (1). All extracts were assayed within 3 weeks of preparation. The same

TABLE I
Amino Acid Content of Plasma from Adult Rats

Amino acid	Group I, non-fasted	Group III, fasted
	<i>γ per ml.</i>	<i>γ per ml.</i>
Leucine	26.6 ± 1.4*	18.5 ± 2.6
Phenylalanine	13.7 ± 0.7	9.4 ± 0.4
Tryptophan	16.8 ± 0.6	11.5 ± 0.8
Valine	26.7 ± 1.1	20.7 ± 2.0
Histidine	9.7 ± 0.4	6.9 ± 0.6
Lysine	58.1 ± 3.7	48.5 ± 1.6
Isoleucine	13.2 ± 1.2	10.9 ± 0.9
Proline	42.7 ± 2.3	23.9 ± 1.1
Tyrosine	22.3 ± 1.6	15.5 ± 1.0
Methionine	9.5 ± 0.1	8.6 ± 0.3
Threonine	44.4 ± 2.8	34.2 ± 1.2
Arginine	32.1 ± 2.6	38.8 ± 0.9
Total	315.8	247.4
Nitrogen, mg. per cent	3.2	2.5

* Standard error of the mean.

composite amino acid standard was employed to determine the concentrations of amino acids in the tissue extracts from all animals.

RESULTS AND DISCUSSION

The mean values together with the standard errors for the free amino acid concentrations in the various rat tissues are shown in Tables I to V. In Table I the concentrations found in the plasma extracts from animals of Groups I and III are presented in order to compare plasma amino acid levels with the levels found in other tissues of the same animals. The plasma values for animals of Group I have been reported in a previous communication (3).

As a result of the short term fast, marked changes in concentrations of several amino acids occurred, while concentrations of other amino acids remained unchanged. Changes in amino acid levels as a result of the short term fast were considered significant only when changes in values of both groups of fasted animals were in the same direction. The levels reported for plasma from fasted rats (Table I, Group III) agree in most instances with the values reported previously by Henderson *et al.* (3). In plasma it has been shown (3) that the concentration of proline was lowered by short periods of fasting. A decrease in this amino acid was also noted in

TABLE II
Amino Acid Content of Liver from Adult Rats

Amino acid	Group I, non-fasted	Group II, fasted	Group III, fasted
	γ per gm.	γ per gm.	γ per gm.
Leucine.....	83.4 \pm 11.9*	108 \pm 7.2	90.5 \pm 6.7
Phenylalanine.....	41.5 \pm 2.7	44.6 \pm 2.5	47.5 \pm 1.8
Tryptophan.....	15.6 \pm 0.8	14.9 \pm 0.7	17.3 \pm 0.5
Valine.....	69.2 \pm 3.6	68.9 \pm 4.4	61.6 \pm 2.4
Histidine.....	79.6 \pm 8.5	77.2 \pm 3.7	48.5 \pm 2.0
Lysine.....	78.3 \pm 1.0	93.4 \pm 4.9	77.2 \pm 7.6
Isoleucine.....	33.6 \pm 2.6	48.5 \pm 4.1	42.3 \pm 2.4
Proline.....	85.4 \pm 2.3	39.4 \pm 3.1	60.8 \pm 2.6
Tyrosine.....	44.0 \pm 3.1	51.9 \pm 3.5	56.4 \pm 1.9
Methionine.....	18.4 \pm 2.6	29.3 \pm 1.4	38.7 \pm 2.4
Threonine.....	99.0 \pm 11.7	117 \pm 8.6	153 \pm 18.6
Arginine.....	13.0 \pm 0.6	11.4 \pm 1.4	10.1 \pm 0.9
Total.....	661.0	704.5	703.9
Nitrogen, mg. per cent.....	6.8	7.2	7.3

* Standard error of the mean.

other tissues (Tables II to V). The other amino acids which decreased in rat tissues during the fasting period were phenylalanine and tyrosine in brain, histidine in muscle, and arginine in spleen. However, the concentrations of leucine, tyrosine, and methionine in liver, leucine in brain, tryptophan in muscle, and possibly threonine in spleen were greater in the rats fasted for 12 hours.

The differences in amino acid values observed in similar tissues of the fasted animals (Groups II and III) cannot be explained. Possibly differences in age, strain, and weight between the two groups of rats influenced the levels of certain amino acids. It should be noted that when

changes occurred during the fast they were generally in the same direction for both groups.

The concentrations of most amino acids varied considerably from one tissue to another. In liver (Table II) the concentrations for most of the free amino acids were 2 to 3 times those found in plasma. Exceptions were histidine, tryptophan, and arginine. Brain amino acid levels (Table III) were found to be about the same or less than those of plasma with the exception of threonine. This amino acid was 3 times more concentrated in brain than in plasma. In the muscle extracts (Table IV) most of the

TABLE III
Amino Acid Content of Brain from Adult Rats

Amino acid	Group I, non-fasted	Group II, fasted	Group III, fasted
	<i>γ per gm.</i>	<i>γ per gm.</i>	<i>γ per gm.</i>
Leucine.....	8.8 ± 0.9*	14.2 ± 1.9	29.8 ± 1.2
Phenylalanine.....	14.6 ± 2.1	8.9 ± 0.9	10.0 ± 1.1
Tryptophan.....	5.2 ± 0.6	3.2 ± 0.2	5.4 ± 0.02
Valine.....	13.6 ± 1.7	11.7 ± 1.1	12.8 ± 0.02
Histidine.....	13.6 ± 1.5	8.6 ± 1.0	12.9 ± 0.5
Lysine.....	31.4 ± 3.1	21.6 ± 2.2	31.9 ± 2.1
Isoleucine.....	8.8 ± 1.5	†	†
Proline.....	13.9 ± 2.7	†	†
Tyrosine.....	20.8 ± 2.2	12.4 ± 1.8	17.9 ± 0.8
Methionine.....	12.2 ± 2.0	8.6 ± 0.9	12.2 ± 0.7
Threonine.....	102 ± 8.5	191 ± 26.0	113 ± 6.4
Arginine.....	23.6 ± 2.0	22.3 ± 2.0	32.5 ± 0.9
Total.....	268.5	302.5	278.4
Nitrogen, mg. per cent.....	2.8	3.3	2.9

* Standard error of the mean.
† These values were so low that they could not be assayed with certainty.

amino acid levels were 2 to 3 times higher than those found in plasma, except for leucine, phenylalanine, tryptophan, histidine, and isoleucine. In spleen (Table V) the concentrations of all amino acids were more than double the respective levels in plasma.

The amounts of α-amino nitrogen calculated from the quantities of amino acids determined in each tissue are included in Tables I to V. The ninhydrin nitrogen in picric acid extracts of rat tissues has been previously reported by Friedberg and Greenberg (5). These workers reported that the postabsorptive concentrations of free amino acid nitrogen were 6.4, 32.1, 43.1, 19.2, and 36.0 mg. per cent, respectively, for plasma, liver, brain, muscle, and spleen. Assuming that tungstic acid extracts of these

TABLE IV
Amino Acid Content of Muscle from Adult Rats

Amino acid	Group I, non-fasted	Group II, fasted	Group III, fasted
	γ per gm.	γ per gm.	γ per gm.
Leucine.....	21.6 \pm 2.2*	22.7 \pm 0.6	30.9 \pm 2.0
Phenylalanine.....	15.5 \pm 0.8	16.0 \pm 0.7	25.2 \pm 1.2
Tryptophan.....	4.8 \pm 0.5	6.0 \pm 0.2	6.7 \pm 0.2
Valine.....	28.1 \pm 2.6	21.1 \pm 1.3	28.6 \pm 1.4
Histidine.....	72.3 \pm 8.1	55.0 \pm 3.2	58.6 \pm 2.4
Lysine.....	88.9 \pm 11.0	65.0 \pm 3.2	116 \pm 8.0
Isoleucine.....	11.5 \pm 1.0	10.3 \pm 0.8	16.2 \pm 1.3
Proline.....	122 \pm 8.5	48.3 \pm 4.7	46.5 \pm 2.4
Tyrosine.....	30.9 \pm 3.3	30.0 \pm 1.8	33.0 \pm 1.3
Methionine.....	17.0 \pm 1.4	15.5 \pm 0.5	15.9 \pm 0.9
Threonine.....	162 \pm 12.7	128 \pm 10.1	154 \pm 6.5
Arginine.....	90.8 \pm 10.7	75.7 \pm 5.8	116 \pm 7.1
Total.....	665.4	493.6	647.6
Nitrogen, mg. per cent.....	6.9	5.0	6.5

* Standard error of the mean.

TABLE V
Amino Acid Content of Spleen from Adult Rats

Amino acid	Group I, non-fasted	Group II, fasted	Group III, fasted
	γ per gm.	γ per gm.	γ per gm.
Leucine.....	104 \pm 8.3*	104 \pm 3.5	87.0 \pm 3.8
Phenylalanine.....	55.2 \pm 4.2	55.8 \pm 3.4	39.5 \pm 2.0
Tryptophan.....	21.0 \pm 1.4	21.6 \pm 1.5	14.5 \pm 0.8
Valine.....	85.8 \pm 6.4	88.7 \pm 6.9	70.6 \pm 3.9
Histidine.....	48.0 \pm 6.7	53.0 \pm 7.6	26.5 \pm 1.9
Lysine.....	94.4 \pm 8.4	91.3 \pm 6.7	79.0 \pm 4.9
Isoleucine.....	43.8 \pm 1.2	48.3 \pm 3.6	41.0 \pm 3.4
Proline.....	117 \pm 4.6	83.9 \pm 7.6	59.9 \pm 5.5
Tyrosine.....	55.5 \pm 1.7	56.9 \pm 2.9	48.4 \pm 2.6
Methionine.....	39.7 \pm 1.5	34.7 \pm 1.1	33.9 \pm 3.5
Threonine.....	120 \pm 4.5	143 \pm 7.1	191 \pm 11.8
Arginine.....	98.4 \pm 1.3	78.2 \pm 3.0	89.9 \pm 2.8
Total.....	882.8	859.4	781.2
Nitrogen, mg. per cent.....	9.1	8.8	8.1

* Standard error of the mean.

tissues contain quantities of α -amino acid nitrogen equivalent to picric acid extracts, the twelve amino acids we have determined account for the

following percentages of the total α -amino acid nitrogen: plasma 45, liver 22, brain 7, muscle 32, and spleen 24. Other tissue constituents not included in our determinations such as glutamine, glutamic acid, glycine, alanine, aspartic acid, serine, hydroxyproline, and cystine probably account for the undetermined quantities of α -amino acid nitrogen. Since the amino acid values presented for each tissue represent only fractional quantities of the total free amino acids, gross changes in the concentrations of any one of the twelve amino acids determined would not be detected by methods by which the amino acids are measured collectively.

Differences in the standard errors of the mean between similar tissues

TABLE VI

*Relative Concentrations of α -Amino Nitrogen in Tissues of Non-Fasted Rats**

Amino acid	Plasma	Liver	Brain	Muscle	Spleen
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Leucine.....	8.90	12.90	3.40	3.36	12.20
Phenylalanine.....	3.64	5.17	4.48	1.92	5.17
Tryptophan.....	3.61	1.57	1.30	0.48	1.59
Valine.....	10.04	12.17	5.88	4.92	11.34
Histidine.....	2.73	10.50	4.40	9.45	4.75
Lysine.....	17.50	11.03	10.90	12.42	9.97
Isoleucine.....	4.43	5.28	3.40	1.79	5.15
Proline.....	16.30	15.20	6.10	21.50	15.60
Tyrosine.....	5.46	5.03	5.85	3.50	4.75
Methionine.....	2.79	2.54	4.15	2.32	4.11
Threonine.....	16.50	17.15	43.30	27.73	15.60
Arginine.....	8.10	1.54	6.86	10.60	9.82

* The relative α -amino nitrogen of each amino acid was calculated as the per cent of the total α -amino acid nitrogen in the respective tissues.

probably indicate the influence of freezing of tissues and short term fasting upon the variation observed between individual animals. A comparison on a percentage basis of the variations in amino acid concentrations between similar tissues of animals from Groups I and II indicates that the variations in muscle were decreased by the short term fast. The freezing treatment appeared to decrease variations in brain, when animals of Groups II and III are compared. Although the results show no conclusive improvement of the variation between analyses for all tissues by the 12 hour fast or by the freezing treatment, the few indications of improvement point to the advisability of such procedures.

The α -amino nitrogen of the individual amino acids in each tissue of rats of Group I was calculated and expressed as per cent of the calculated total α -amino nitrogen in the respective tissues. These percentages (Table

VI) enable comparisons of the relative α -amino nitrogen concentration in plasma, liver, brain, muscle, and spleen of the rat for the twelve amino acids determined. It can be seen in Table VI that there is good agreement between the patterns for liver and spleen. However, the respective patterns for plasma, brain, and muscle are not comparable to each other nor to those of liver and spleen.

SUMMARY

1. The concentrations of twelve amino acids in tungstic acid extracts of rat plasma, liver, brain, muscle, and spleen have been determined by the microadaptation of the microbiological assay.

2. The characteristic free amino acid levels for individual animals receiving an 18 per cent casein ration *ad libitum* and for two groups of individual animals in the postabsorptive state have been presented.

BIBLIOGRAPHY

1. Schurr, P. E., Thompson, H. T., Henderson, L. M., and Elvehjem, C. A., *J. Biol. Chem.*, **182**, 29 (1950).
2. Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, **109**, 657 (1935).
3. Henderson, L. M., Schurr, P. E., and Elvehjem, C. A., *J. Biol. Chem.*, **177**, 815 (1949).
4. Henderson, L. M., Brickson, W. L., and Snell, E. E., *J. Biol. Chem.*, **172**, 31 (1948).
5. Friedberg, F., and Greenberg, D. M., *J. Biol. Chem.*, **168**, 405 (1947).

THE INFLUENCE OF FASTING AND NITROGEN DEPRIVATION ON THE CONCENTRATION OF FREE AMINO ACIDS IN RAT TISSUES*

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In a previous paper (1) we reported a method for the determination of twelve free¹ amino acids by the microbiological procedure in rat liver, brain, muscle, and spleen. In the present paper we have employed this method to study the influence of fasting and nitrogen deprivation on the concentration of free amino acids in these tissues.

EXPERIMENTAL

Sprague-Dawley male rats, weighing from 250 to 300 gm., were maintained on a stock ration for at least 2 weeks. The rats were then given a purified ration (2) for 2 weeks, after which they were divided into uniform groups of three animals. During the experimental period that followed, one group, the control, received the purified ration *ad libitum*; four groups were fasted for 1, 3, 5, or 7 days respectively; and six groups were deprived of the nitrogen for 1, 3, 5, 7, 14, or 21 days. The nitrogen-free ration was prepared by replacing the casein of the purified ration with an equal quantity of sucrose. The tissue extracts were prepared and analyzed as previously described (1).

RESULTS AND DISCUSSION

The effect of fasting and nitrogen deprivation on the free amino acid concentrations in rat liver, muscle, spleen, and brain are shown graphically in Figs. 1 to 4. The control values for unfasted animals agree in most instances with the average values for unfasted individual rats reported in an earlier paper (3). It is recognized that the animals of the

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¹ Free as used throughout implies microbiologically available amino acids and may include certain combined forms as well as free amino acids.

control group did not represent a specific state with regard to food absorption, since they may or may not have eaten just prior to the preparation of the tissues for analysis.

Changes in Amino Acid Concentration As Result of Fasting—In the tis-

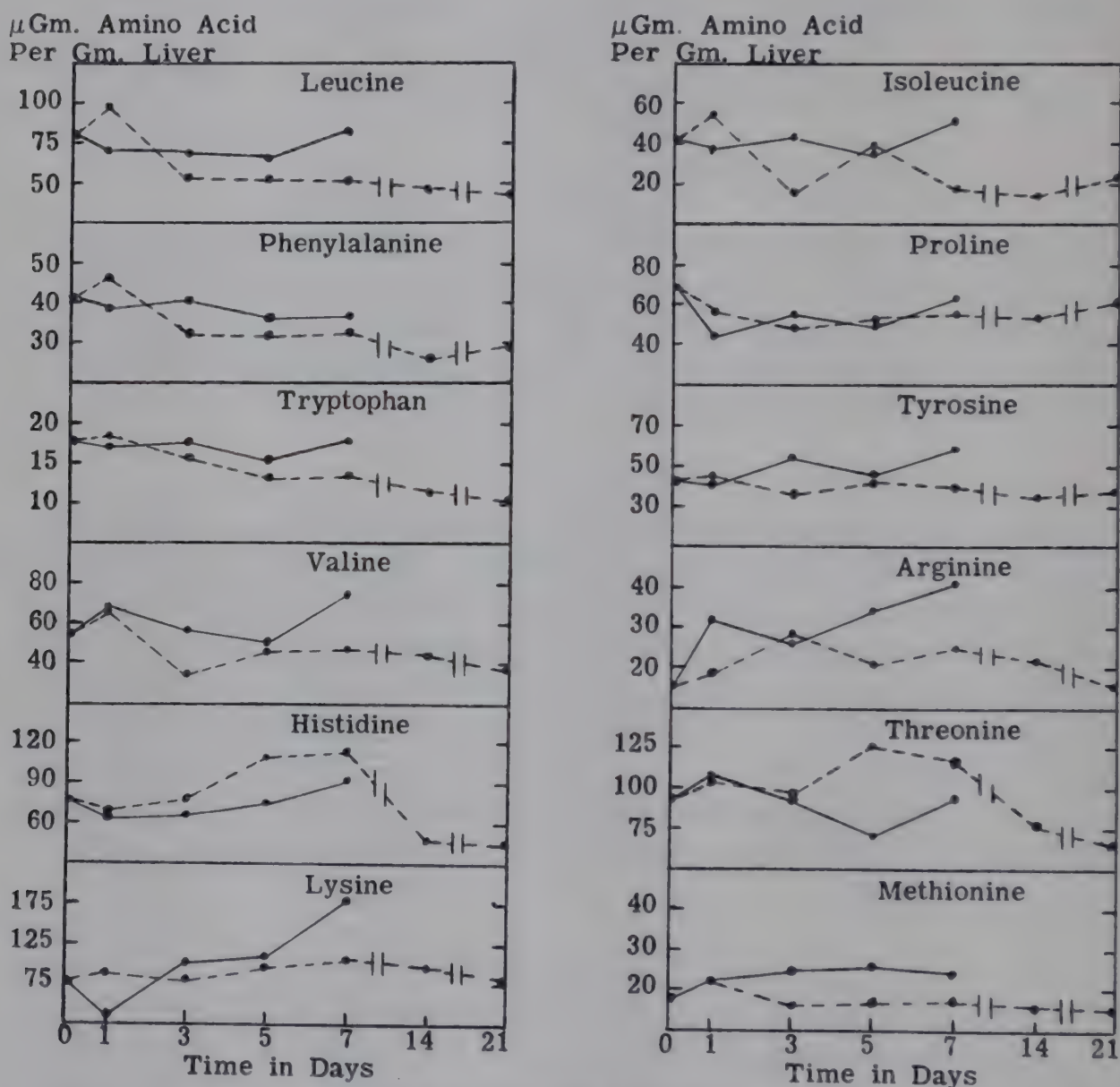


FIG. 1. The effect of fasting and nitrogen deprivation on the concentration of free amino acids in rat liver. The broken line represents the results from animals on nitrogen deprivation; the solid line, results from animals on total fasting.

sues studied, the changes in amino acids as a result of fasting did not follow a general pattern. Although a number of the amino acids behaved similarly in a given tissue, a single amino acid did not always follow the same trend in all the tissues. Especially large changes were observed, in many cases, as a result of the first day of fasting. Henderson *et al.* (2) found similar changes in the plasma amino acids.

For the fasting studies reported here, it was generally observed that the amino acid concentrations were equal to or greater than the control values by the end of the fast. On the other hand, the concentrations of a few amino acids were subnormal at the end of the experimental period. These include proline in all the tissues; lysine in muscle, spleen, and brain; histi-

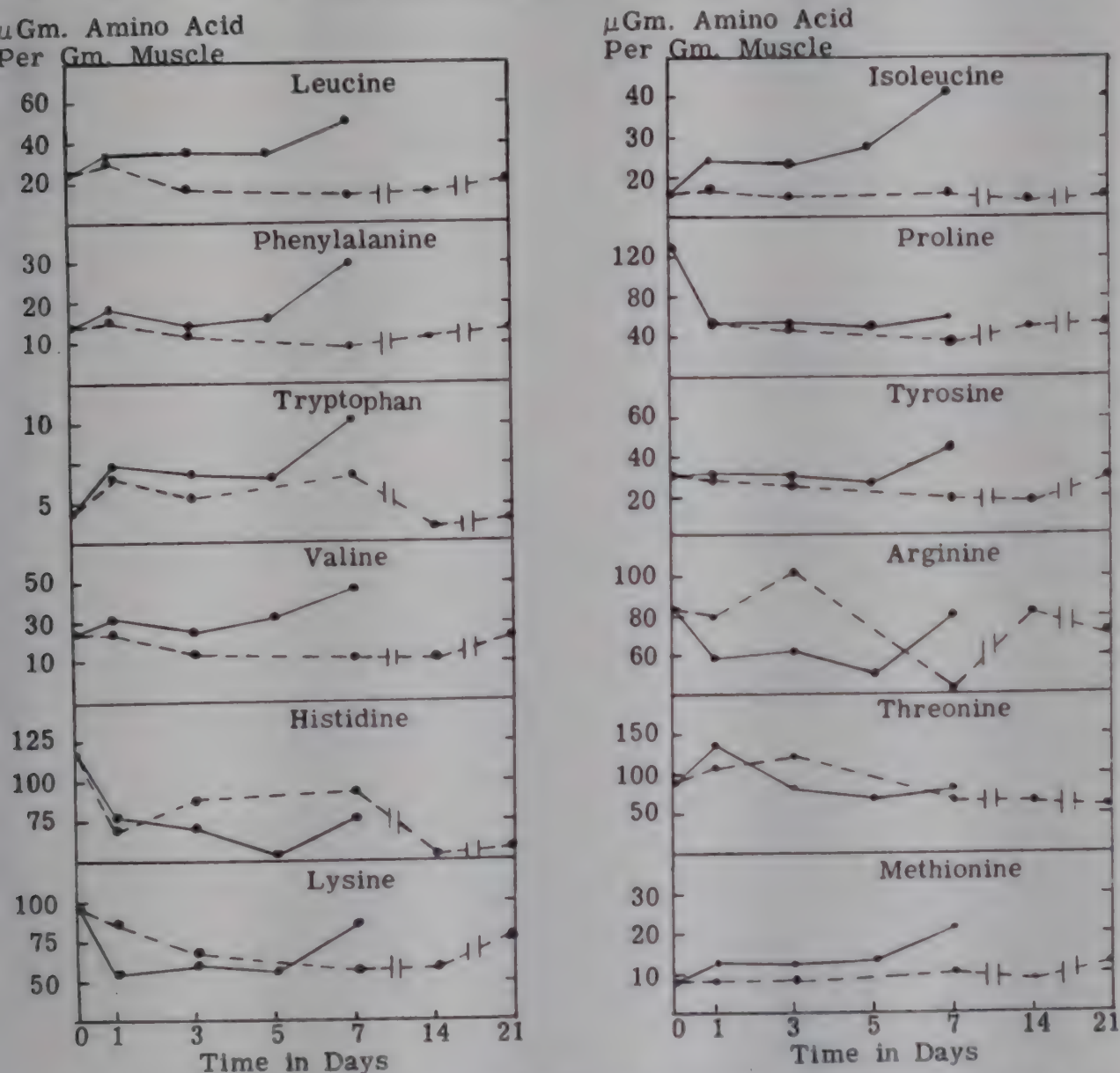


FIG. 2. The effect of fasting and nitrogen deprivation on the concentration of free amino acids in rat muscle. The broken line represents the results from animals on nitrogen deprivation; the solid line, results from animals on total fasting.

dine in muscle and brain; valine and phenylalanine in brain; and threonine in spleen.

Changes in Amino Acid Concentrations As Result of Nitrogen Deprivation—In animals deprived of protein the changes in free amino acids were different from those occurring in fasted animals. Except for some increases the 1st day, the concentrations of amino acids remained constant

or tended to decrease in all the tissues studied. A few amino acids varied from this pattern in that they increased above the control values before dropping to lower levels. These include histidine in all tissues, arginine in muscle, liver, and spleen, and threonine in muscle and liver.

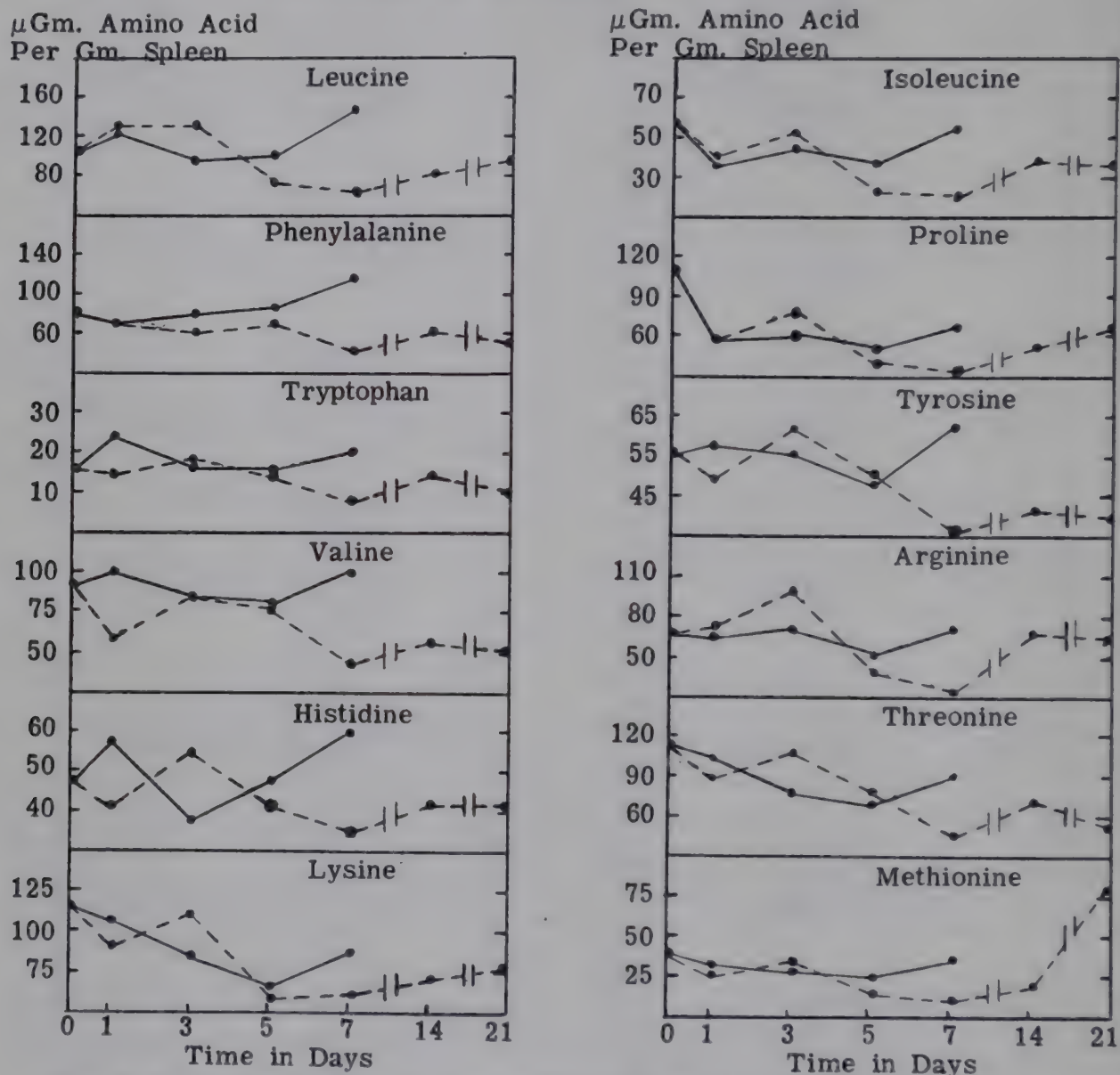


FIG. 3. The effect of fasting and nitrogen deprivation on the concentration of free amino acids in rat spleen. The broken line represents the results from animals on nitrogen deprivation; the solid line, results from animals on total fasting.

In muscle and liver the amino acid concentrations reached a minimum value by the end of the 2nd week and then remained more or less constant. On the other hand, in brain and spleen the minimum concentrations were reached by the end of the 7th day. The concentration then remained relatively constant with the exception of methionine in spleen and proline in brain. The concentration of these amino acids increased significantly during the 3rd week.

It was interesting to note that the concentration of proline was significantly decreased in most cases by the dietary conditions imposed. In the tissues studied the slight to moderate increases in amino acid concentrations observed as fasting progressed are in accord with the observations that amino nitrogen tends to increase during fasting (4-6). Since amino

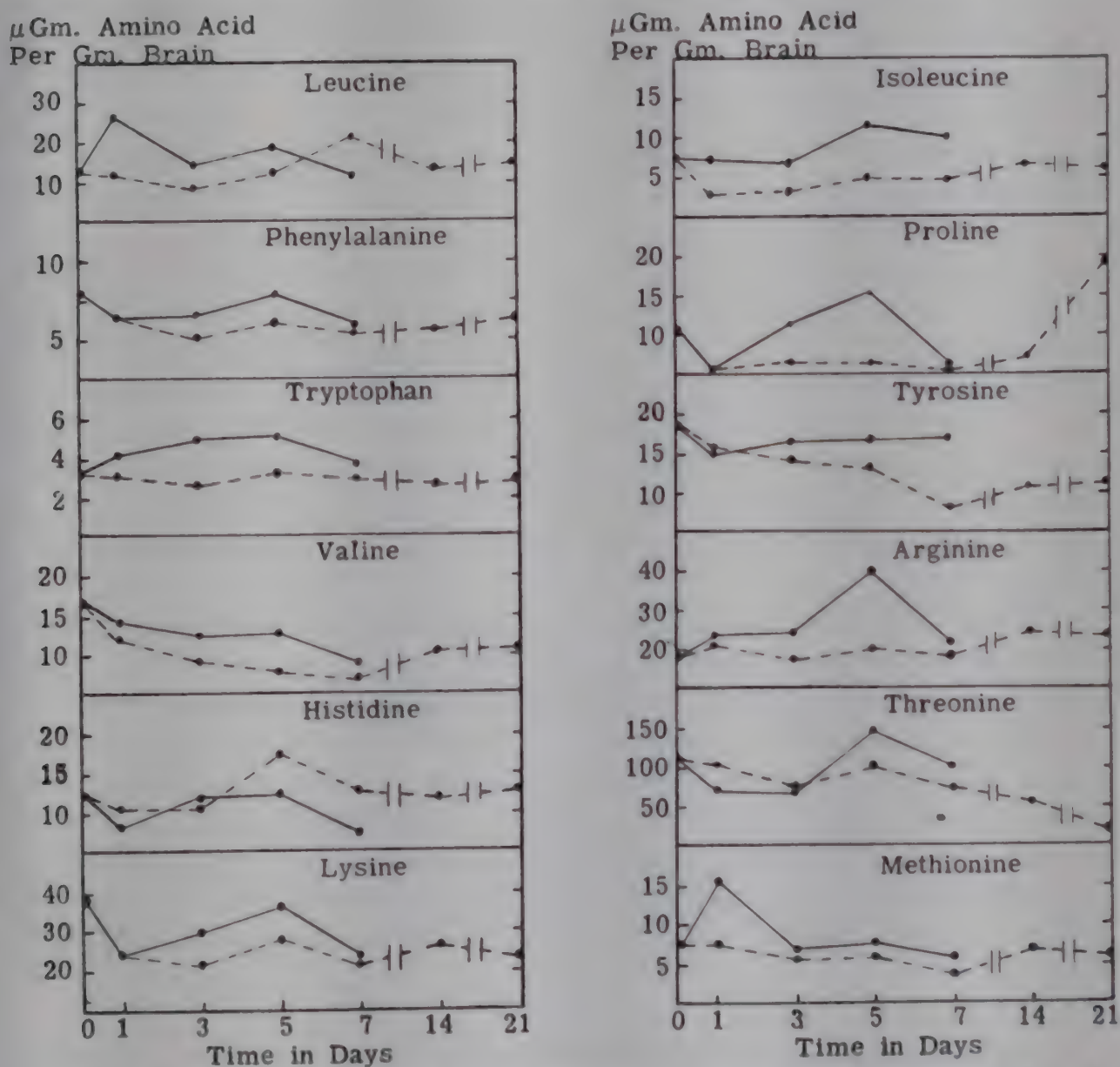


FIG. 4. The effect of fasting and nitrogen deprivation on the concentration of free amino acids in rat brain. The broken line represents the results from animals on nitrogen deprivation; the solid line, results from animals on total fasting.

acids are the products of protein breakdown, the increase in amino acid concentration during periods of protein breakdown is not surprising.

In the animals on a protein-free diet the concentration of amino acids tends to decrease as the protein deficiency becomes more acute. These results appear to be contrary to those noted by Luck (7) and Mitchell *et al.* (6) who have reported that the amino nitrogen remains constant in

the tissues of animals on a protein-free diet. The two results are not necessarily in conflict, since the undetermined amino nitrogen in this investigation may change in such a way that the total amino nitrogen remains constant.

In this investigation the sum of the concentrations of the twelve amino acids determined was less during protein deprivation than during fasting. This may have been the result of less rapid destruction of the proteins. The weight loss of the protein-deprived animals averaged 17 per cent as compared with 25 per cent for the rats fasted for 7 days. Henderson *et al.* (2) have reported values of 13 per cent and 31 per cent weight losses for nitrogen-deprived and fasted animals respectively. Similar losses have been reported by Roche (8).

A second experiment was performed about 6 months later. It differed from the first only in that three control groups were used. The average control values obtained were in excellent agreement with the control values of the first experiment. This good agreement between experiments was not observed during fasting and nitrogen deprivation except for phenylalanine, tryptophan, isoleucine, proline, tyrosine, and arginine. The concentrations of the other amino acids were, in general, higher during the second experiment. However, the same trends occurred in both experiments.

The data presented here for fasted and protein-deprived rats supplement the plasma data published earlier (2). The values may not represent absolute concentrations, since small amounts of peptides which are active for the test organism may be present in the tissue extracts. It is also recognized that concentrations expressed in terms of fresh tissue weight are not entirely satisfactory, since changes in other tissue components as a result of the experimental conditions imposed might affect the values obtained. However, the values presented here may be useful for comparison purposes in other depletion and negative nitrogen balance studies.

SUMMARY

A microadaptation of the microbiological assay procedure has been used to measure the concentration of twelve amino acids in tungstic acid extracts of rat liver, muscle, spleen, and brain during various periods of fasting and nitrogen deprivation. The changes in concentrations of the individual amino acids in the tissues have been discussed.

BIBLIOGRAPHY

1. Schurr, P. E., Thompson, H. T., Henderson, L. M., and Elvehjem, C. A., *J. Biol. Chem.*, **182**, 29 (1950).

2. Henderson, L. M., Schurr, P. E., and Elvehjem, C. A., *J. Biol. Chem.*, **177**, 815 (1949).
3. Schurr, P. E., Thompson, H. T., Henderson, L. M., Williams, J. N., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, **182**, 39 (1950).
4. Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, **16**, 231 (1913-14).
5. Buglia, G., and Constantion, A., *Arch. ital. biol.*, **60**, 51 (1913).
6. Mitchell, H. H., Nevens, W. B., and Kendall, F. E., *J. Biol. Chem.*, **52**, 417 (1922).
7. Luck, J. M., *J. Biol. Chem.*, **77**, 13 (1928).
8. Roche, A., *Compt. rend. Acad.*, **197**, 279 (1933).

THE INFLUENCE OF CHILLING AND EXERCISE ON FREE AMINO ACID CONCENTRATIONS IN RAT TISSUES*

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Speculation has arisen concerning the relation of chilling and exercise to inception and effect of virus diseases. It has been observed in bacteriophage studies (1) that amino acids play an important rôle in virus adsorption and production. In studying the effect of temperature on virus adsorption by *Escherichia coli* B, Anderson (2) observed that the decreased adsorption of virus T₄ at low temperatures is returned to normal by addition of L-tryptophan to the medium. His tests also indicate that isoleucine, leucine, methionine, and norleucine are active for other T strains. The importance of tryptophan in virus infection of animals has been demonstrated by Kearney *et al.* (3).

Because of the indicated importance of amino acids in virus diseases, we have undertaken a study of changes in free¹ amino acid concentrations in various tissues of the rat under conditions which are believed to aid virus infection in animals: chilling and exhaustive exercise.

EXPERIMENTAL

Eighteen male, albino rats of the Holtzman strain weighing 210 ± 10 gm. were given a synthetic casein ration (4) for 2 weeks before use in the experimental studies. The experimental procedure was planned so that six animals served as controls, six animals were chilled, and six animals were exercised. Two animals from each of these groups were carried through the regimen and sacrificed on each of 3 days. The control animals were fasted for 18 hours before sacrifice; they were deprived of water for the last 6 hours. The animals to be chilled were fasted for 12 hours and then kept without water in a cold room at -5° for 6 hours. The animals to be exercised were fasted for 12 hours, then placed in a treadmill

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¹ Free as used throughout implies microbiologically available amino acids and may include combined forms as well as free amino acids.

(20 cm. diameter), and exercised continuously at the rate of 15 revolutions per minute for 6 hours.

Immediately after the fasting, chilling, or exercise periods, the animals were anesthetized with sodium amytal; tungstic acid extracts of plasma, liver, muscle, and brain of the animals were prepared as previously described (4-6). The excised tissues were frozen in dry ice immediately after removal from the animals. Similar tissues from two animals of each group were pooled in preparing the tungstic acid extracts. After the eighteen animals had been sacrificed and the tissue extracts prepared, the extracts were assayed for microbiologically available amino acids at duplicate levels of 0.04, 0.06, and 0.08 ml. levels (5, 7).

TABLE I
Free Amino Acid Concentrations in Plasma

Amino acid	Control	Chilled	Exercised
	γ per ml.	γ per ml.	γ per ml.
Leucine.....	31.4 \pm 2.2*	38.1 \pm 3.7	52.2 \pm 1.5
Phenylalanine.....	12.7 \pm 0.1	14.1 \pm 0.7	15.6 \pm 0.8
Tryptophan.....	13.5 \pm 1.2	14.7 \pm 0.8	8.3 \pm 1.1
Valine.....	28.4 \pm 0.6	32.6 \pm 1.4	48.0 \pm 3.0
Histidine.....	11.5 \pm 0.6	9.2 \pm 0.5	11.4 \pm 1.0
Lysine.....	44.6 \pm 3.0	30.4 \pm 2.5	46.5 \pm 2.5
Isoleucine.....	19.2 \pm 0.4	18.1 \pm 0.5	17.5 \pm 1.6
Proline.....	20.3 \pm 1.1	5.5 \pm 1.3	3.5 \pm 1.7
Tyrosine.....	16.9 \pm 0.8	14.0 \pm 1.5	15.3 \pm 0.5
Methionine.....	9.9 \pm 0.2	5.3 \pm 0.2	5.7 \pm 0.5
Threonine.....	48.2 \pm 3.1	30.9 \pm 1.7	35.9 \pm 2.9
Arginine.....	39.1 \pm 1.0	31.0 \pm 2.7	29.6 \pm 1.2

* Standard error of the mean.

Results

The results of the amino acid determinations for the various tissue extracts of the three groups of animals are presented in Tables I to IV. The standard errors for the amino acid determinations are also given. Changes in chilled or exercised animals were considered significant only if the differences were well outside the standard error ranges.

Of the four tissues studied, the plasma amino acids (Table I) appeared to show the greatest changes when the animals were chilled or exercised. Since the changes in the plasma might very well reflect total changes in other tissues of the animals, this may account for the greater differences observed in plasma than in the other tissues. The most outstanding change in plasma amino acids occurred with proline, which decreased

greatly when the animals were either chilled or exercised. Other amino acids that decreased in plasma during both chilling and exercise were

TABLE II
Free Amino Acid Concentrations in Liver

Amino acid	Control	Chilled	Exercised
	γ per gm.	γ per gm.	γ per gm.
Leucine.....	66.7 \pm 0.5*	73.1 \pm 3.8	105 \pm 2.8
Phenylalanine.....	34.5 \pm 0.7	39.4 \pm 3.0	39.2 \pm 2.0
Tryptophan.....	11.6 \pm 0.2	11.2 \pm 0.3	12.3 \pm 0.7
Valine.....	44.6 \pm 4.6	56.9 \pm 2.8	109 \pm 6.1
Histidine.....	87.0 \pm 9.2	46.3 \pm 3.6	49.9 \pm 1.9
Lysine.....	58.9 \pm 12	69.1 \pm 14	128 \pm 15
Isoleucine.....	28.0 \pm 1.1	33.0 \pm 1.7	37.4 \pm 1.9
Proline.....	56.0 \pm 2.6	56.3 \pm 0.3	67.8 \pm 3.2
Tyrosine.....	45.7 \pm 2.6	41.6 \pm 3.2	51.9 \pm 0.2
Methionine.....	24.7 \pm 0.7	26.5 \pm 2.2	29.6 \pm 1.1
Threonine.....	148 \pm 20	162 \pm 6.8	202 \pm 16
Arginine.....	†	†	†

* Standard error of the mean.

† These values were so low that they could not be assayed with certainty.

TABLE III
Free Amino Acid Concentrations in Muscle

Amino acid	Control	Chilled	Exercised
	γ per gm.	γ per gm.	γ per gm.
Leucine.....	43.3 \pm 2.2*	42.4 \pm 2.8	69.6 \pm 7.3
Phenylalanine.....	25.4 \pm 1.3	25.8 \pm 2.5	32.8 \pm 0.4
Tryptophan.....	7.6 \pm 0.4	9.2 \pm 0.8	10.0 \pm 1.2
Valine.....	41.0 \pm 1.3	40.1 \pm 2.6	72.7 \pm 1.9
Histidine.....	104 \pm 14	96.0 \pm 2.0	104 \pm 9.1
Lysine.....	52.0 \pm 6.0	53.4 \pm 1.5	57.5 \pm 6.4
Isoleucine.....	30.7 \pm 1.7	27.1 \pm 0.8	35.8 \pm 0.8
Proline.....	43.9 \pm 4.3	33.9 \pm 2.5	37.3 \pm 1.0
Tyrosine.....	36.3 \pm 0.7	34.2 \pm 3.0	43.4 \pm 1.2
Methionine.....	24.9 \pm 1.8	19.0 \pm 1.0	23.8 \pm 2.4
Threonine.....	115 \pm 13	102 \pm 21	108 \pm 9.0
Arginine.....	68.7 \pm 6.0	59.0 \pm 10	66.7 \pm 0.1

* Standard error of the mean.

methionine, threonine, and arginine, while tryptophan decreased only during exercise and lysine only during chilling. Leucine, phenylalanine, and valine on the other hand increased during chilling and exercise. It

is interesting to note that the effect of chilling tended to be in the same direction as that of exercise, though the effect was not quite as pronounced. This held true in most cases not only for plasma but for all the tissues studied.

In the liver (Table II) the majority of amino acids increased in concentration during exercise with the exception of histidine which decreased markedly. Again the amino acid values for the chilled animals appeared to lie between those for the control and the exercised animals.

In muscle (Table III) definite changes occurred in the concentrations of leucine, phenylalanine, tryptophan, and valine after exercise. During chilling no significant changes occurred in muscle.

TABLE IV
Free Amino Acid Concentrations in Brain

Amino acid	Control	Chilled	Exercised
	γ per gm.	γ per gm.	γ per gm.
Leucine.....	20.0 \pm 1.0*	20.5 \pm 0.9	25.1 \pm 1.5
Phenylalanine.....	9.2 \pm 0.6	11.2 \pm 0.4	9.3 \pm 1.0
Tryptophan.....	4.8 \pm 0.3	6.7 \pm 0.7	4.3 \pm 0.1
Valine.....	17.8 \pm 0.5	19.5 \pm 1.2	21.0 \pm 0.3
Histidine.....	17.4 \pm 1.7	18.2 \pm 2.0	7.5 \pm 0.5
Lysine.....	27.8 \pm 1.5	32.3 \pm 3.7	30.0 \pm 2.0
Isoleucine.....	6.3 \pm 1.5	4.8 \pm 0.8	6.4 \pm 0.9
Proline.....	14.2 \pm 1.4	13.6 \pm 1.6	14.5 \pm 0.9
Tyrosine.....	17.3 \pm 0.6	17.0 \pm 0.4	15.0 \pm 0.3
Methionine.....	12.0 \pm 0.9	8.7 \pm 0.8	8.5 \pm 0.3
Threonine.....	132 \pm 14	111 \pm 6.2	112 \pm 4.9
Arginine.....	33.8 \pm 0.9	27.9 \pm 2.6	19.5 \pm 1.8

* Standard error of the mean.

The free amino acid concentrations in brain (Table IV) appeared to be less affected by chilling and exercise than the amino acids in the other tissues. The most marked changes occurred in the concentrations of histidine and arginine, which decreased during exercise.

In all of the tissues studied leucine and valine increased during exercise. Otherwise, no consistent changes were observed, even when the amino acids were classified as glucogenic, ketogenic, or essential and non-essential.

The relation of the observed changes in amino acid concentrations to virus problems is not evident at the present time. More work on the metabolism of free amino acids needs to be done before the results reported in this paper can be completely interpreted. It is hoped that our results

will give suggestions for further research on problems relating to free amino acid metabolism.

SUMMARY

A study of changes in free amino acid concentrations in tissues of the rat after chilling and exercise has been made. Although marked changes were observed, direct implication of the results with virus infection or amino acid metabolism cannot be made at present.

BIBLIOGRAPHY

1. Cohen, S. S., *Bact. Rev.*, **13**, 1 (1949).
2. Anderson, T. F., *J. Bact.*, **55**, 659 (1948).
3. Kearney, E. B., Pond, W. L., Plass, B. A., Maddy, K. H., Elvehjem, C. A., and Clark, P. F., *J. Bact.*, **55**, 89 (1948).
4. Schurr, P. E., Thompson, H. T., Henderson, L. M., Williams, J. N., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, **182**, 39 (1950).
5. Schurr, P. E., Thompson, H. T., Henderson, L. M., and Elvehjem, C. A., *J. Biol. Chem.*, **182**, 29 (1950).
6. Henderson, L. M., Schurr, P. E., and Elvehjem, C. A., *J. Biol. Chem.*, **177**, 815 (1949).
7. Henderson, L. M., Brickson, W. L., and Snell, E. E., *J. Biol. Chem.*, **172**, 31 (1948).

MUTANT STRAINS OF NEUROSPORA DEFICIENT IN AMINATING ABILITY

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Since the inauguration of biochemical work on *Neurospora crassa*, mutant strains have been found which have requirements for most of the commonly occurring L- α -amino acids (1). In many cases the requirement seems to be for the keto analogue of the amino acid, since the keto analogue and the D-amino acid, if the latter is attacked by the D-amino acid oxidase (2), are generally utilized. One exception to this is the case of the *lysineless* mutant for which L- and D- α -aminoadipic acids are active but α -ketoadipic is inactive (3).

This paper deals with two mutant strains which will grow on any one of a number of α -amino acids and which appear to require exogenous α -amino nitrogen.

Mutant Strains

Mutant strains 32213 and 47305 were recorded as responding to a number of different amino acids, and the dissection of asci from outcrosses to wild type showed that each very probably involved a single locus mutation. A novel feature of these mutants was their apparent ability to grow on minimal medium (Fries, Medium 3) at 35° but not at 25° (initial tests and genetic data due to M. B. Houlahan, unpublished). In experiments of the present writer these strains have been found to start growing slowly on minimal medium after 4 to 5 days at 25° and after 2 to 3 days at 35°, the temperature effect being on the rate of adaptation. This adaptation to growth on minimal medium will not persist through a vegetative transfer, and it seems clear that back-mutation is not involved.

Crosses of strains 47305A \times 32213a made by the writer failed to yield any wild type cultures from five completely dissected asci and thirty-one random ascospores. This, taken in conjunction with the qualitatively identical growth requirements of the two strains, seems to be strong evidence that the two mutations are at the same locus.

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Methods

Except where otherwise stated, growth responses were measured as dry weight after 72 hours at 25° in 20 ml. of the usual Fries Medium 3 (minimal), appropriately supplemented, in 125 ml. Erlenmeyer flasks. In some experiments a nitrogen-free minimal medium was used, differing from the Fries Medium 3 in the omission of ammonium nitrate and the substitution of potassium tartrate for ammonium tartrate. Inoculations were made with drops of suspensions of conidia in sterile water.

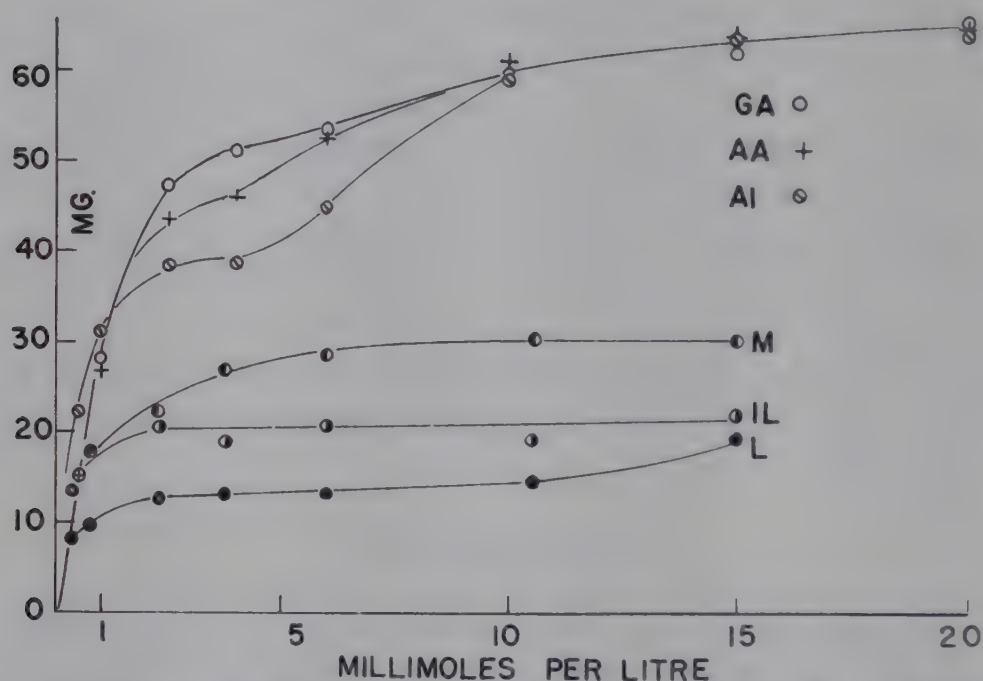


FIG. 1. Growth response of strain 32213 to L-glutamic acid (GA), L-aspartic acid (AA), DL-alanine (AL), L-methionine (M), L-isoleucine (IL), and L-leucine (L). Alanine is plotted as concentrations of the L isomer.

Responses of Mutant Strains to Amino Acids

The only amino acids enabling strain 32213 to grow as well as the wild type were glutamic acid, aspartic acid, and alanine. The optimal concentration of these amino acids was about M/67 of the L isomer, an unusually high requirement. DL-Ornithine was somewhat more active than DL-alanine at the lower concentrations but became inhibitory at a concentration of more than 0.002 M of the L isomer. Other amino acids were less active at all concentrations than the first three mentioned, and gave growth-concentration curves reaching a maximum at a much lower concentration (Fig. 1). Strain 47305 responded better than strain 32213 to all amino acids, and arginine, ornithine, or proline, at appropriate concentrations, supported growth as well as the wild type. L-Proline was exceptional in giving an S-shaped growth-concentration curve with both

mutants, being rather inactive at low concentrations and comparatively very active at higher concentrations. No explanation can be advanced for this at the present time. Maximal growth of strain 47305 was obtained with 0.004 M glutamic acid. The response of the two strains to two concentrations of the amino acids, and other substances, is shown in Table I. The data come from different but closely comparable experiments.

As shown in Table I, the only two α -keto acids tested, pyruvic and α -ketoglutaric acids, were quite inactive. Experiments were done to determine whether any of the D-amino acids could support growth. The results are given in Table II. The D isomers of the six amino acids listed are evidently inactive, and D-aspartic acid, D-glutamic acid, and probably D-alanine are inhibitory. Since extracts of strain 32213 mycelium have been shown by the writer to possess D-amino acid oxidase activity (DL-glutamic acid, DL-aspartic acid, D L-leucine, and the L isomers, have been tested), the non-utilization of D-amino acids by this mutant may indicate its inability to aminate the keto acid analogues. *Leucineless* and *methionineless* mutants have been shown to use both the D isomers and the corresponding α -keto acids (2).

Utilization of Ammonia

The inability of strains 32213 and 47305 to aminate α -keto acids might be expected to result in an impaired uptake of ammonia by these strains. That this is the case has been shown by experiments in which mutant and wild type mycelia were grown in flasks containing 20 ml. of N-free minimal medium with ammonium nitrate (200 γ per ml.) added, and a limiting concentration of DL-alanine or L-glutamic acid. Mycelia were harvested at intervals and weighed, and the pH and ammonia content of the medium determined. The latter was done by distillation of the ammonia by the Conway-Byrne technique, followed by nesslerization. The color was measured in a Klett-Summerson photoelectric colorimeter. The results of a typical experiment are shown in Fig. 2.

The initial rise in pH and ammonia concentration in the case of the mutant may be attributed to a reduction of nitrate to ammonia, which proceeds faster than the ammonia can be used up. In the case of the wild type, the pH and ammonia concentration drop from the first, indicating that ammonia is used up faster than it is produced from nitrate. Another experiment, with mutant 47305 and the wild type strain E5256 gave essentially the same results. In this experiment it was found that increasing the L-glutamic acid concentration to 0.01 M (an optimal concentration for strain 47305) had a strong sparing effect on the uptake of ammonia by the wild type, and a much smaller such effect on the mutant.

TABLE I

Response of Mutants to Amino Acids and Related Compounds

72 hours growth in 20 ml. of medium. Acidic media adjusted to about pH 5 with NaOH. The concentrations given are those of the L isomers.

The results are expressed in mg. of dry weight of the mycelium.

Substance added	Strain 47305		Strain 32213	
	0.001 M	0.004 M	0.001 M	0.004 M
DL-Ornithine*	42	56	23	31
DL-Alanine	37	59	24	30
L-Glutamic acid	33*	76*	23	41
L-Aspartic "		57	23	36
L-Arginine	30	67	20	24
L-Proline	4	58	1*	11*
DL-Methionine		37	16	21
DL-Leucine		36	5	10
DL-Valine		38	7	13
DL-Isoleucine		34	11	19
DL-Norleucine		39	7	13
DL- α -Aminobutyric acid†				4
DL- α -Aminocaprylic acid†				0
DL-Norvaline†				3
DL-Phenylalanine		27	15	15
L-Tryptophan		20 (ca.)	12	14
L-Cysteine*	5		0	0
L-Cysteic acid* (1)			0	0
DL- α -Amino- δ -hydroxyvaleric acid* (2)	4	3	0	
DL-Citrulline*	0	0	0	0
DL-Threonine		0	0	0
DL-Homoserine†	0			
DL-Serine		0	0	0
L-Lysine		0	0	0
L-Histidine		0	0	0
Glycine			0	0
D- α -Aminoadipic acid‡ (3)			0	
Aminofumaric diamide* (3)			0	
α -Oximinoglutaric acid*			0	0
Pyruvic acid oxime* (4)			0	0
α -Ketoglutaric acid*	0	0	0	0
Pyruvic acid*			0	0
Succinic acid*		0		0
Urea*			0	0

The compounds indicated were kindly supplied by (1) Mr. J. L. Reissig, (2) Dr. E. E. Snell, (3) Dr. H. K. Mitchell, (4) Mr. B. S. Strauss.

* Sterilized by filtration; other compounds autoclaved with minimal medium at 15 pounds for 5 minutes.

† Tested at 0.025 M of L isomer.

‡ Tested at 5 mg. in 20 ml.

TABLE II

Comparison of Activities of L- and DL-Amino Acids

72 hours growth in 20 ml. of medium. The concentrations of amino acids are given in terms of the L isomer. All weights are averages from duplicate flasks.

Experiment No.	Amino acid	Dry weight of strain 32213		
		0.00125 M	0.0025 M	0.005 M
		mg.	mg.	mg.
1	L-Alanine	34.2	42.4	45.7
1	DL-Alanine	30.0	34.2	41.0
2	L-Glutamic acid	47.2		59.2
2	DL-Glutamic acid	25.0		46.2
2	L-Aspartic acid	43.7		60.8
2	DL-Aspartic acid	30.0		41.0
3	L-Methionine	12.6	15.2	17.4
3	DL-Methionine	12.2	13.9	18.3
3	L-Leucine	10.8	12.2	
3	DL-Leucine	10.2	10.7	
3	L-Isoleucine	7.9	10.0	11.3
3	DL-Isoleucine	6.6	8.6	11.3

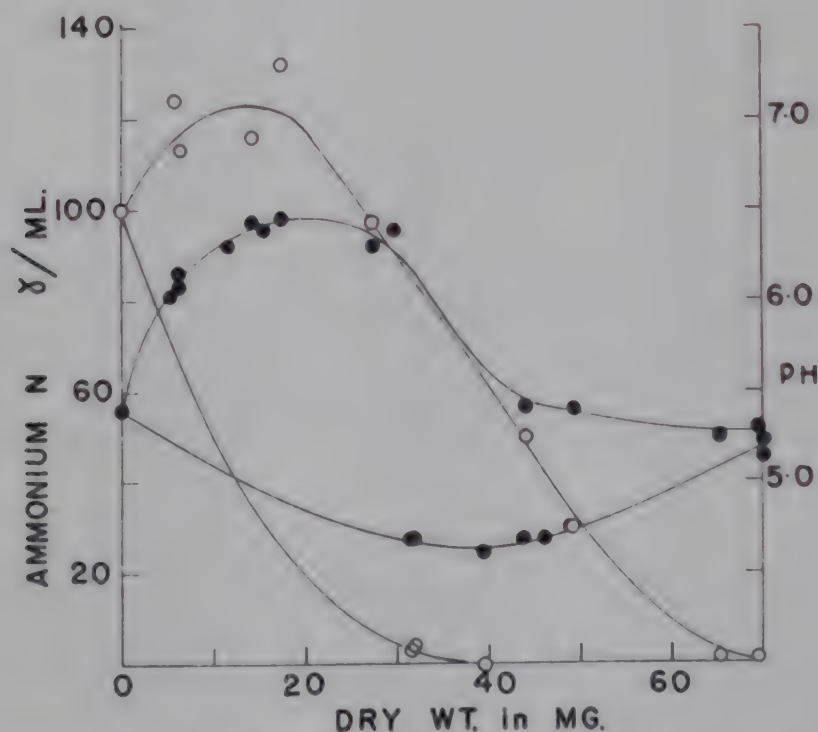


FIG. 2. Changes in pH and ammonia content of the medium during growth of strain 32213 and of wild type strain P4/12. ●, pH; ○, ammonia concentration by nesslerization. The upper curves in each case are for strain 32213, and the lower for the wild type. L-Glutamic acid added, 3 mg. per 20 ml.

That the ammonia accumulated during the early stages of growth of strain 32213 comes from nitrate is made almost certain by an experiment

in which mycelial pads of this strain, grown on a glutamic acid medium, were washed and transferred to N-free minimal medium with and without added potassium nitrate and sucrose. The flasks were incubated at 25° with shaking, and the ammonia content of the media estimated at intervals. The results are shown in Fig. 3. It appears that, in the presence of sugar, strain 32213 mycelia reduce nitrate with the accumulation of ammonia. This does not, of course, mean that the reduction necessarily proceeds at the inorganic level.

It is interesting to note that, although strains 32213 and 47305 take up ammonia more slowly than the wild types, they do eventually use it

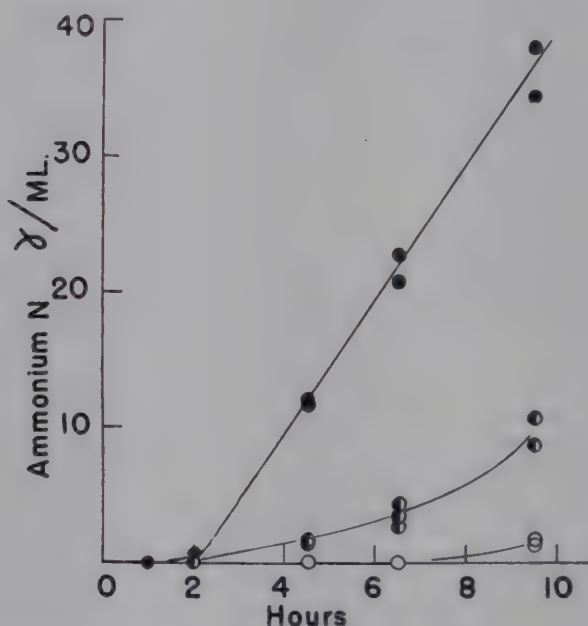


FIG. 3. Ammonia production by mycelium of strain 32213. 20 ml. lots of N-free minimal medium, with indicated supplements, shaken at 25° with strain 32213 mycelia. ●, 0.5 per cent sucrose and 0.5 per cent KNO₃ added; ◐, 0.5 per cent KNO₃ only; ○, 0.5 per cent sucrose only. Dry weights of mycelia at the start of the experiment, about 32 mg. (from control); at the end, sucrose-nitrate 70 mg., sucrose only 69 mg., nitrate only 33 mg.

completely. This may be due both to utilization of ammonia for processes other than amination of α -keto groups to make amino acids (*e.g.* formation of amide groups), and to a slow "leaking" at the amination block. That this block is not always complete is shown by the growth of these mutants on minimal medium after 4 to 5 days.

Ammonium or nitrate ions added to N-free minimal medium plus glutamic acid have a stimulatory effect for the wild type, and generally for the mutants also (see, for example, Table III).

Inhibitory Amino Acids

Of the amino acids listed as inactive in Table I, glycine, DL-serine, L-histidine, and DL-threonine were found to inhibit growth of the mutant

32213 at concentrations at which the wild type was practically unaffected. The wild type was inhibited considerably more by these amino acids when

TABLE III
Growth Inhibition of Strain 32213 and Wild Type

All weights are averages of duplicate flasks. Experiments on minimal and N-free minimal media done at different times; 72 hours incubation.

Additions	Dry weight			
	Strain 32213		Strain E5256 (wild type)	
	Minimal	N-free minimal	Minimal	N-free minimal
	mg.	mg.	mg.	mg.
0.02 M DL-alanine	53		73	
Same + L-histidine 0.005 M	35		68	
“ + “ 0.01 M	30		65	
“ + DL-serine 0.005 M	61		88	
“ + “ 0.01 M	39		85	
“ + DL-threonine 0.005 M	45		83	
“ + “ 0.01 M	42		80	
0.01 M L-glutamic acid	61	37	87	52
Same + L-histidine 0.005 M	1	8	64	8
“ + “ 0.01 M	0	7	59	6
“ + DL-serine 0.005 M	0	2	78	30
“ + “ 0.01 M	0	1	71	26
“ + DL-threonine 0.005 M	8	19	75	40
“ + “ 0.01 M	3	12	67	34
0.01 M L-glutamic acid + 0.01 M DL-alanine		31		44
Same + L-histidine 0.005 M		26		18
“ + “ 0.01 M		24		16
“ + DL-serine 0.005 M		39		52
“ + “ 0.01 M		42		51
“ + DL-threonine 0.005 M		46		62
“ + “ 0.01 M		51		64
0.01 M L-aspartic acid	53		81	
Same + L-histidine 0.005 M	0		64	
“ + “ 0.01 M	0		55	
“ + DL-serine 0.005 M	0		74	
“ + “ 0.01 M	0		67	
“ + DL-threonine 0.05 M	4		73	
“ + “ 0.01 M	3		67	
0.02 M L-glutamic acid	76	50	83*	55
Same + glycine 0.025 M	2	2	61*	10
“ + “ 0.05 M	0	0	51*	9

* Wild type strain P4/12.

supplied with L-glutamic acid as sole source of nitrogen in N-free minimal medium. These experiments are summarized in Table III.

The situation is most clear in the case of histidine inhibition. Here the wild type is inhibited as much as the mutant in the absence of inorganic nitrogen. The presence of the ammonium tartrate and ammonium nitrate in the Fries Medium 3 largely prevents inhibition of the wild type, but not of the mutant. The same appears to be true for glycine, serine, and threonine inhibition, though here inhibition of the wild type is less complete than that of the mutant, even in the absence of inorganic nitrogen. This may be regarded as further evidence that inorganic nitrogen is unavailable to the mutant for some reaction.

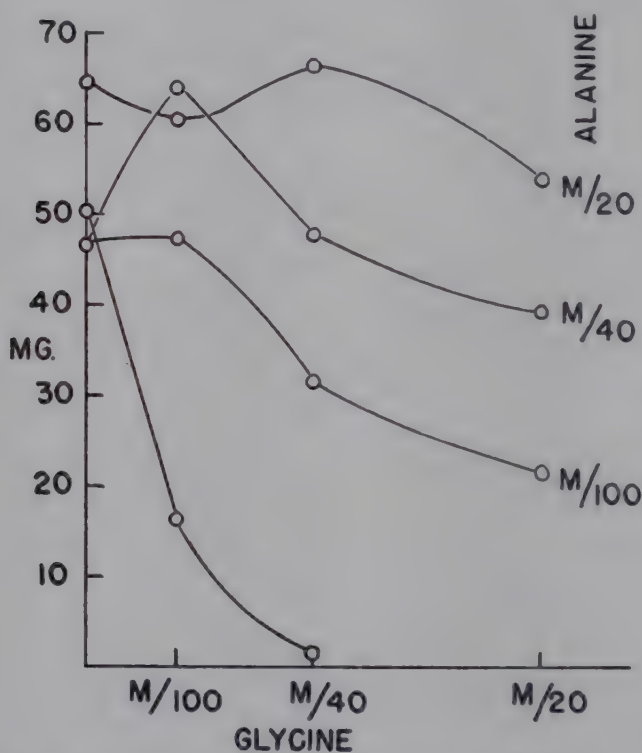


FIG. 4. Release of inhibition of strain 32213 by DL-alanine. 72 hours growth on N-free minimal medium with 0.02 M L-glutamic acid. pH adjusted to 5.0. Concentrations of alanine expressed as amounts of the L isomer.

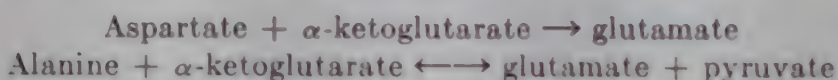
It will be seen from Table III that the presence of alanine prevents the inhibition of both mutant and wild type by DL-serine and DL-threonine, and to a great extent prevents inhibition by L-histidine at the concentrations tested. The relation between alanine and glycine, at least, appears to be a competitive one (see Fig. 4). Inhibition by glycine is overcome by an approximately equimolar amount of L-alanine.

Of the other amino acids which will not support growth, DL-citrulline and DL- α -amino- δ -hydroxyvaleric acid are inhibitory for mutant 32213 when supplied together with a limiting amount of L-glutamic acid. L-Cysteine also inhibits both strain 32213 and the wild type at concentrations greater than 0.002 M. L-Lysine, on the other hand, does not

inhibit the wild type or strain 32213 at 0.01 M, and the same applies to L-cysteic acid.

Experiments on Transamination

The growth responses of mutants 32213 and 47305 to amino acids suggested the existence in *Neurospora* of a number of transaminase systems effecting the transfer of amino groups among all the amino acids supporting growth of the mutants. The system pyruvate + glutamate \leftrightarrow alanine + α -ketoglutarate in *Neurospora*, activated by pyridoxal phosphate, has recently been demonstrated in this laboratory (B. S. Strauss, unpublished). The present writer has been able to demonstrate that powdered lyophilized strain 32213 mycelium, suspended in M/15 phosphate buffer at pH 7.1, was able to carry out the reactions



This was done by running ascending phenol-water paper chromatograms (4) of reaction mixtures deproteinized at zero time and after 1 to 2 hours at 35°. The appearance of a spot corresponding to the newly formed amino acid and a decrease in the intensity of the spot of the original amino acid were clearly demonstrable in the presence, but not in the absence, of the appropriate α -keto acid. Lyophilized mycelium which had been kept for 1 month had very low activity, which was much increased by the addition of pyridoxal phosphate¹ at the rate of 4 γ per ml. It was not possible to show formation of glutamate from α -ketoglutarate with leucine, methionine, valine, or norleucine as amino donor by this qualitative method, and an apparent small effect with ornithine was of doubtful significance.

Thus *Neurospora* appears to possess the transaminase systems which have been demonstrated in bacteria (5), animal tissues (6), and higher plants (7).

Experiments on Succinic Acid-Requiring Mutants

If it is assumed that the reaction primarily blocked in strains 32213 and 47305 is the amination of α -ketoglutaric acid or oxalacetic acid to give glutamic or aspartic acid, the high activity of these two amino acids, and that of alanine, would be due to the presence of the transaminase systems mentioned above. No very convincing demonstration of transamination between ornithine and glutamic acid was obtained, however, and the high activities of ornithine, proline, and arginine may be due to the conversion of their carbon skeletons to glutamic acid. Proline and ornithine have been shown to be convertible to glutamic acid in the rat (8, 9). It

¹ Kindly supplied by Dr. H. K. Mitchell.

appeared that a way of checking this possibility was to test the activities of these amino acids on succinic acid-requiring mutants. This class of mutants will grow if supplied with succinic, malic, or fumaric acid, or with glutamic or aspartic acid (10). It was found by the writer that the last two compounds are practically inactive at concentrations lower than 0.002 M, but supported normal growth at 0.01 M. Succinic acid, on the other hand, supported appreciable growth at a concentration of 0.0005 M, but was less effective than glutamic or aspartic acid at 0.01 M. For strain 47403, 0.001 M glutamic acid was inactive by itself, but almost doubled

TABLE IV

Response of Succinic Acid-Requiring Strains to Proline and Ornithine

72 hours incubation. Proline and ornithine were sterilized by filtration and added to the medium after autoclaving.

Additions to minimal medium	Dry weight		
	Strain E5256 (wild type)	Strain 35501	Strain 47403
	mg.	mg.	mg.
0.001 M succinic acid	43.0	21.7	14.7
	46.0	21.4	14.4
	46.0	23.0	14.3
0.001 M succinic acid + L-proline 0.0025 M	49.6	30.5	17.3
	51.4	33.0	18.3
	49.8	29.9	18.3
0.001 M succinic acid + DL-ornithine 0.005 M	47.6	26.6	20.0
	49.4	26.5	23.1
	49.6		21.7
0.001 M succinic acid + L-glutamic acid 0.0025 M			37.1
			37.4

the growth when added as a supplement to 0.001 M succinic acid. DL-Ornithine alone was found to be inactive for strain 47403.

An experiment was carried out to determine whether L-proline or DL-ornithine could be utilized by the succinic acid mutants 35501 and 47403 in the presence of limiting succinate. The results are shown in Table IV. Although the significance of these data is made doubtful by the slight stimulatory effect of proline and ornithine on the wild type E5256, the data are consistent with a slow utilization of these amino acids, although proline and ornithine are much less active than glutamic acid in this respect. If this is true, it means that the carbon skeletons of proline and ornithine can be converted to a dicarboxylic acid, probably via glutamic acid.

DISCUSSION

The evidence presented makes it seem clear that the mutant strains 32213 and 47305 are unable to synthesize the amino groups for a wide range of amino acids from ammonia. If the one gene-one reaction hypothesis holds, the fact that a single mutation has this effect implies that there is one primary reaction by which amino groups for these amino acids are made from ammonia. Analogy with *Escherichia coli* (11), yeast (12), and animal tissues (13) strongly suggests that this reaction is α -ketoglutarate + ammonia \rightarrow glutamate, and that the mutants have a defective glutamic dehydrogenase system. Attempts to find this system in *Neurospora* wild type have so far failed, but are being continued.

The fact that any one of a number of amino acids will support growth of the mutants suggests that transamination mechanisms, in the sense of transfer of an amino group from one carbon skeleton to another without the formation of ammonia as an intermediate, may link all these amino acids, with the possible exceptions of ornithine, arginine, and proline, which may give glutamic acid directly. Rautanen (14) reports the existence in the pea of valine \rightarrow glutamate and leucine \rightarrow glutamate transaminations with low activity. These, and other undemonstrated transaminating systems, are probably of low activity in *Neurospora* compared with the glutamate \leftrightarrow aspartate and glutamate \leftrightarrow alanine systems. This is in keeping with the comparatively low activities for mutant 32213 of amino acids other than ornithine and the three demonstrably involved in active transamination. The failure of a number of amino acids to support growth may be due to their low effectiveness as amino donors combined with their inhibitory properties. The fact that lysine, which is not inhibitory at the concentrations used, is inactive, is not surprising in view of the fact that the nitrogen of lysine is not exchanged with the amino groups of other, dietary, amino acids in the rat (15, 16). The inactivity of cysteic acid is perhaps surprising, since it has been shown to be a substrate for animal transaminase (17). It is known that *Neurospora* can normally convert citrulline to arginine (18), and it seems very probable that it can convert α -amino- δ -hydroxyvaleric acid to ornithine (unpublished work of the author). These reactions can evidently not be effected by the amination-deficient mutants. This is to be expected in the case of the citrulline \rightarrow arginine conversion, since this reaction has, in animal tissues, been shown to require aspartic acid as an amino donor (19, 20). It is readily conceivable that aspartic acid, or some other amino acid, is necessary for the amination step from aminohydroxyvaleric acid to ornithine.

It seems possible from the experiments on succinic acid-requiring mutants that proline and ornithine can be converted to glutamic acid in

Neurospora. This would help to account for the relatively high activities of these amino acids for mutants 32213 and 47305, but aminohydroxyvaleric acid is an unlikely intermediate, since it is practically inactive. Since evidence obtained by the writer (to be published separately) indicates that α -amino- δ -hydroxyvaleric acid may be an intermediate in proline and ornithine formation from glutamic acid, the syntheses of proline and ornithine by this route may not be directly reversible.

SUMMARY

1. The mutant strains 32213 and 47305 of *Neurospora crassa*, which almost certainly represent mutations at the same locus, will grow if supplied with any one of a number of amino acids.

2. Glutamic acid, aspartic acid, alanine, and ornithine are the most active in supporting growth. Arginine, proline, methionine, isoleucine, norleucine, valine, leucine, phenylalanine, and tryptophan follow in roughly decreasing order of activity. Cysteine, glycine, serine, threonine, homoserine, α -amino- δ -hydroxyvaleric acid, lysine, histidine, and citrulline are inactive.

3. All α -keto acids and D-amino acids tested were inactive.

4. Mutants 32213 and 47305, in contrast to the wild type, tend to accumulate ammonia in a medium containing nitrate.

5. In a medium containing L-glutamic acid as the sole nitrogen source, glycine, DL-serine, L-histidine, and DL-threonine strongly inhibit both strain 32213 and the wild type. The inhibitions of the wild type, but not of the mutant, are released by the addition of inorganic nitrogen. All these inhibitions are ineffective in the presence of alanine at sufficiently high concentrations.

6. *Neurospora* possesses the two transaminase systems which have been established in other organisms. No good enzymatic evidence for other transaminases in *Neurospora* has been obtained.

7. Experiments with succinic acid-requiring mutants indicate that ornithine and proline will support growth to some extent in the presence of limiting succinic acid, suggesting that they can be converted to glutamic acid.

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The author is indebted to many at the Kerckhoff Laboratories, especially Dr. N. H. Horowitz, for much helpful discussion and criticism.

BIBLIOGRAPHY

1. Horowitz, N. H., *Advances in Genetics*, in press.
2. Horowitz, N. H., *J. Biol. Chem.*, **154**, 141 (1944).

3. Mitchell, H. K., and Houlahan, M. B., *J. Biol. Chem.*, **174**, 883 (1948).
4. Williams, R. J., and Kirby, H., *Science*, **107**, 481 (1948).
5. Lichstein, H. C., and Cohen, P. P., *J. Biol. Chem.*, **157**, 85 (1945).
6. Green, D. E., Leloir, L. F., and Nocito, V., *J. Biol. Chem.*, **161**, 559 (1945).
7. Leonard, M. J. K., and Burris, R. H., *J. Biol. Chem.*, **170**, 701 (1947).
8. Roloff, M., Ratner, S., and Schoenheimer, R., *J. Biol. Chem.*, **136**, 561 (1940).
9. Stetten, M. R., and Schoenheimer, R., *J. Biol. Chem.*, **153**, 113 (1944).
10. Lewis, R. W., *Am. J. Bot.*, **35**, 292 (1948).
11. Adler, E., and Hellström, V., *Z. physiol. Chem.*, **255**, 14 (1938).
12. Adler, E., Gunther, G., and Everett, J. E., *Z. physiol. Chem.*, **255**, 27 (1938).
13. Dewan, J. G., *Biochem. J.*, **32**, 1378 (1938).
14. Rautanen, N., *Ann. acad. sc. Fennicae, Series A*, **33**, 1 (1948).
15. Schoenheimer, R., Ratner, S., and Rittenberg, D., *J. Biol. Chem.*, **127**, 333 (1939).
16. Ratner, S., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **134**, 653 (1940).
17. Cohen, P. P., *J. Biol. Chem.*, **136**, 565 (1940).
18. Srb, A. M., and Horowitz, N. H., *J. Biol. Chem.*, **154**, 129 (1944).
19. Ratner, S., and Pappas, A., *J. Biol. Chem.*, **179**, 1183 (1949).
20. Ratner, S., and Pappas, A., *J. Biol. Chem.*, **179**, 1199 (1949).

NON-UTILIZATION OF α -AMINO- ϵ -UREIDO-*n*-CAPROIC ACID, PIPERIDINE-2-CARBOXYLIC ACID, AND α -AMINOADIPIC ACID FOR GROWTH IN RATS ON A LYSINE-DEFICIENT DIET*

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The experiments of Schoenheimer and coworkers (1, 2), which indicated that the pathway of catabolism of lysine in the rat may be unique among the amino acids, have stimulated a considerable interest in the study of lysine metabolism. In considering this problem, one possibility which has occurred to us was that lysine in its metabolism might, at least in some species, be analogous to ornithine, of which it is the next higher homologue.

The known interrelationships of ornithine with the amino acids arginine, citrulline, proline, and glutamic acid (2-5) suggested the study of the next higher homologues of each of these amino acids. Since the present experiments were begun, one of these homologues, α -aminoadipic acid, has been isolated from a natural source (6) and has been shown to be an intermediary product in lysine metabolism (7, 8).

As a first step in the study of the possible biochemical significance of these amino acid homologues, the compounds were synthesized and tested for their ability to support growth in rats on a lysine-deficient diet. The preparation of α -amino- ϵ -guanidino-*n*-caproic acid (homoarginine) and evidence for its conversion to lysine in the rat have already been reported (see (20)).

The preparation of α -amino- ϵ -ureido-*n*-caproic acid (homocitrulline) was considered by Kurtz (9) and has been mentioned in the patent literature (10). In the present study, the L isomer was readily prepared from L-lysine, the general procedure for the preparation of citrulline from ornithine being used (9). Piperidine-2-carboxylic acid, the homologue of proline, has been prepared by Ost (11) and others. In the present study, it was conveniently prepared by catalytic hydrogenation of picolinic acid.

The various homologues have been tested for their effects on growth in rats on a lysine-deficient diet. Since this work was completed, Geiger

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and Dunn (12) have reported the failure of α -aminoadipic acid to support growth of rats on a lysine-deficient diet.

EXPERIMENTAL

Preparation of L- α -Amino- ϵ -ureido-n-caproic Acid (L-Homocitrulline)—The method was patterned after the procedure of Kurtz (9) for the preparation of citrulline. 20 gm. of L-lysine monohydrochloride were converted to the copper derivative by being boiled for 45 minutes with 10 gm. of cupric oxide in 150 ml. of water. The suspension was filtered and 32 gm. of urea were added to the filtrate. The resulting solution was evaporated to 0.5 volume and then heated under reflux for 4 hours. The paste was cooled, and the solid collected and thoroughly washed with water. The copper salt was decomposed with hydrogen sulfide, the copper sulfide removed with the aid of decolorizing carbon, and the filtrate evaporated to dryness *in vacuo*. The residue was recrystallized from 50 ml. of hot water by addition of 100 ml. of ethanol, yielding 12 gm. (60 per cent) of material. Further recrystallization yielded a product melting at 212–213° (decomposition) (uncorrected); $[\alpha]_D^{23} = +6.4^\circ$ (4 per cent in water); C 44.44, H 7.93; calculated for $C_7H_{15}O_3N_3$, C 44.20, H 8.00 per cent.

The compound was further characterized by conversion to the monobenzoyl derivative by a customary procedure (13). Recrystallization from water gave a product melting at 175–176° (uncorrected); $[\alpha]_D^{23} = -8.9^\circ$ (1 per cent in 95 per cent ethanol); neutralization equivalent 294, N 13.81; calculated for $C_{14}H_{19}O_4N_3$, neutralization equivalent 293, N 14.33 per cent.

Tests with *Streptococcus faecalis* and *Leuconostoc mesenteroides*, according to the assay procedure for lysine (14), indicated that the compound possessed very slight activity. It is not certain whether this activity represents inherent activity of the compound, slight contamination with lysine, or slight conversion to lysine during autoclaving.

Preparation of DL-Piperidine-2-carboxylic Acid Hydrochloride (Homoproline)—To 25 gm. of picolinic acid hydrochloride (15) dissolved in 60 ml. of water was added 0.5 gm. of platinum oxide catalyst (16), and the mixture was shaken for 12 hours under a pressure of 2 atmospheres of hydrogen. The catalyst was then reactivated by shaking in air and the hydrogenation continued for another 12 hours. The product was recovered by removal of catalyst, evaporation of the solution, and recrystallization of the residue from 100 ml. of ethanol and 40 ml. of benzene, yielding 14 gm. (55 per cent). Further recrystallization from alcohol-benzene yielded material which melted at 258–261° (uncorrected), compared to values of 259–261° and 264° previously reported (17, 18); C 43.61, 43.43, H 7.31, 7.05; calculated for $C_6H_{12}O_2NCl$, C 43.63, H 7.27.

A portion of the product was converted to the benzoyl derivative which was recrystallized from benzene-ligroin, m.p. 126-127° (uncorrected); neutralization equivalent 229, N 6.09; calculated for $C_{13}H_{15}O_3N$, neutralization equivalent 233, N 6.01 per cent.

TABLE I
Feeding Experiments with Rats on Lysine-Deficient Basal Diet

Rat No.	Initial weight	Average daily food intake	Average daily weight change	Dietary supplement
	gm.	gm.	gm.	per cent
1B	40	7.6	+2.7	1.0 L-lysine monohydrochloride
2B	51	6.8	+2.6	1.0 " "
3B	40	6.3	+2.4	0.5 " "
4B	57	8.0	+2.6	0.5 " "
5B	48	7.8	+2.9	0.5 " "
6B	49	5.4	+0.8	0.25 " "
7B	53	5.7	+0.7	0.25 " "
8B	61	9.4	+0.7	0.25 " "
9B	53	5.5	-0.3	None
10B	68	4.4	-0.5	"
11B	60	4.7	-0.1	"
12B	72	6.5	+1.1	0.5 L-lysine monohydrochloride + 3.0 L-homo-citrulline
13B	60	6.5	+1.5	" "
14B	52	6.5	+1.1	0.25 L-lysine monohydrochloride + 3.0 L-homo-citrulline
15B	59	6.4	+1.0	" "
16B	60	6.6	+0.9	" "
17B	62	4.8	-0.2	3.0 L-homocitrulline
18B	61	4.3	-0.3	3.0 "
19B	55	6.3	+1.8	0.5 L-lysine monohydrochloride + 3.0 DL-piperidine-2-carboxylic acid
20B	57	6.7	+1.8	" "
21B	46	5.5	+0.9	0.25 L-lysine monohydrochloride + 3.0 DL-piperidine-2-carboxylic acid
22B	59	5.3	+0.5	" "
23B	61	5.7	+0.8	" "
24B	55	3.7	-0.3	3.0 DL-piperidine-2-carboxylic acid
25B	64	3.2	-0.4	3.0 "

When tested with *S. faecalis* and *L. mesenteroides*, DL-piperidine-2-carboxylic acid gave no growth response in a lysine-free medium or in a medium containing suboptimal amounts of lysine.

α-Aminoadipic Acid—This compound was prepared according to the procedure of Waelsch *et al.* (19). It gave correct elementary analyses.

The compound failed to support the growth of *S. faecalis* or *L. mesenteroides* on a lysine-free medium or to affect the growth obtained from suboptimal amounts of lysine. This is in agreement with the findings of Geiger and Dunn (12). Furthermore, DL- α -aminoadipic acid failed to support growth of *L. mesenteroides* on a medium free from aspartic acid or to alter the growth response from suboptimal amounts of aspartic acid. Likewise, experiments with *L. arabinosus* on a medium free of glutamic acid showed no growth response to the compound. Thus, it may be concluded that, under the conditions of these experiments, the organisms tested are unable to convert α -aminoadipic acid to glutamic acid or aspartic acid.

Feeding Experiments—In one series of trials, twenty-five weanling albino male rats (Sprague-Dawley) were placed on the lysine-deficient diet previously described (20), different groups receiving supplements of L-lysine, L- α -amino- ϵ -ureido-*n*-caproic acid, and DL-piperidine-2-carboxylic acid. The experiments were conducted in the same general way as in the previous study. All animals were placed on a supplement of 1 per cent of L-lysine monohydrochloride for a preliminary period of 9 days. They were then divided into groups and fed the various supplements indicated in Table I for a period of 20 days. From the results recorded in Table I, it can be seen that animals receiving no dietary supplement consistently lost weight, those receiving 0.25 per cent of L-lysine grew consistently but slowly, while those receiving higher levels of lysine showed reasonably good growth. Addition of L- α -amino- ϵ -ureido-*n*-caproic acid or DL-piperidine-2-carboxylic acid to the basal diet exerted no appreciable effect on growth. When either of the compounds was added to a diet containing suboptimal amounts of L-lysine, no significant increase in the rate of growth resulted. Additional feeding experiments with both compounds, including an experiment with L- α -amino- ϵ -ureido-*n*-caproic acid at a level of 1.5 per cent in the diet, have confirmed these results, and indicate that neither compound can support growth in rats on a lysine-deficient diet.

Experiments of a similar nature on rats in which DL- α -aminoadipic acid was fed both in the absence of lysine and in the presence of suboptimal amounts of lysine showed entirely negative effects. Since Geiger and Dunn (12) have already reported similar findings, the present results are not presented in detail.

SUMMARY

L- α -Amino- ϵ -ureido-*n*-caproic acid (homocitrulline) has been prepared by a convenient synthesis.

L- α -Amino- ϵ -ureido-*n*-caproic acid, DL-piperidine-2-carboxylic acid, and DL- α -aminoadipic acid, higher homologues of citrulline, proline, and glutamic acid, respectively, have been tested for their ability to support the

growth of rats on a lysine-deficient diet. Under the conditions of the experiments, none of the compounds showed any ability to support growth.

BIBLIOGRAPHY

1. Schoenheimer, R., *The dynamic state of body constituents*, Cambridge, 2nd edition (1946).
2. Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **158**, 71 (1945).
3. Womack, M., and Rose, W. C., *J. Biol. Chem.*, **171**, 37 (1947).
4. Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, **210**, 33 (1932).
5. Srb, A. M., and Horowitz, N. H., *J. Biol. Chem.*, **154**, 129 (1944).
6. Blass, J., and Macheboeuf, M., *Helv. chim. acta*, **29**, 1315 (1946).
7. Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H., *J. Biol. Chem.*, **173**, 423 (1948).
8. Mitchell, H. K., and Houlahan, M. B., *J. Biol. Chem.*, **174**, 883 (1948).
9. Kurtz, A. C., *J. Biol. Chem.*, **122**, 477 (1937-38).
10. British patent 421407 (1934); *Chem. Abstr.*, **29**, 3468 (1935).
11. Ost, H., *J. prakt. Chem.*, **27**, 287 (1883).
12. Geiger, E., and Dunn, H. J., *J. Biol. Chem.*, **178**, 877 (1949).
13. Carter, H. E., and Stevens, C. M., *J. Biol. Chem.*, **138**, 627 (1941).
14. Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, **172**, 15 (1948).
15. *Org. Syntheses*, **20**, 79 (1940).
16. *Org. Syntheses*, coll. **1**, 463 (1941).
17. Mende, F., *Ber. chem. Ges.*, **29**, 2887 (1896).
18. Willstätter, R., *Ber. chem. Ges.*, **29**, 389 (1896).
19. Waelsch, H., Owades, P., Miller, H. K., and Borek, E., *J. Biol. Chem.*, **166**, 273 (1946).
20. Stevens, C. M., and Bush, J. A., *J. Biol. Chem.*, in press.

STUDIES ON ETHIONINE

I. INHIBITION OF PROTEIN SYNTHESIS IN INTACT ANIMALS

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Since the original demonstration by Dyer (1) that ethionine (α -amino- γ -ethylthiolbutyric acid) fails to support the growth of rats on a methionine-deficient diet, it has been shown by several groups of workers that this homologue of methionine also inhibits the growth of various microorganisms. The inhibition is relieved by methionine (2-4).

The present experiments were designed to test the possibility that ethionine inhibits protein synthesis in animal tissues. The work was facilitated by the use of methionine labeled with radioactive sulfur which is rapidly incorporated into the proteins both of intact animals and of tissue slices. It has been shown that the uptake of radioactive methionine is inhibited by ethionine *in vivo* and that the inhibition is relieved by methionine under the proper conditions.

It has also been found that ethionine inhibits the conversion of methionine to cystine, which is tentatively taken to indicate that ethionine also interferes with the demethylation of methionine; that is, homocysteine formation is inhibited by ethionine.

The incorporation into protein not only of methionine but also of glycine is inhibited by ethionine. This observation appears to be of some significance with respect to the theory of protein synthesis.

EXPERIMENTAL

Methionine—Methionine-S³⁵ was synthesized by the methods previously described (5). The unlabeled methionine used was the DL isomer unless otherwise stated.

Ethionine—For part of the ethionine used, we are obliged to United States Industrial Chemicals; the remainder was synthesized either from methionine or from homocystine by a procedure which is essentially that used by Dyer (1). The preparation proceeds more rapidly from homocystine than from methionine because of the greater ease with which homocystine is reduced by sodium in liquid ammonia compared with the slow

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demethylation of methionine by the same reagents. After the addition of ethyl iodide to the reaction mixture, the preparation is allowed to stand for 15 minutes, then a few ml. of ethyl alcohol are added. The ethionine is isolated as described by Dyer. It contains less than 1 per cent homocystine (or homocysteine) as impurity. The yield from lots of 100 gm. of homocystine in 2 liters of liquid ammonia is 85 per cent.

Glycine—We are indebted to Dr. Tolbert and Dr. Calvin for the synthesis of the carboxyl-labeled glycine used in these experiments.

Animals—The virgin femal rats used in the *in vivo* studies were of the Long-Evans strain and weighed between 170 to 210 gm. They were fasted 12 hours before the start of the experiments. The mice used were males of the A strain weighing 22 to 27 gm. and were likewise fasted 12 hours before the start of the experiments. In every experiment three animals were used to establish each result.

Procedure

Rats—Following the 12 hour fast the experimental animals were subjected to a treatment approximately as follows: beginning, 75 mg. of ethionine; 2 hours, 25 mg. of ethionine; 2.17 hours, tracer dose of labeled methionine; 3 hours, 50 mg. of ethionine; 4.75 hours, animal sacrificed. All substances were given by intraperitoneal injection and the control animals received an equal volume of water. The tracer dose of methionine used varied between 6.7 and 13.4 μM with 2.5×10^5 to 4.6×10^6 counts per minute, depending on the particular experiment. In those experiments in which an attempt was made to relieve the inhibition of methionine incorporation resulting from the injection of ethionine, the controls received amounts of methionine equal to those given the experimental animals. This is made necessary because of the reduction in the uptake of the labeled methionine due to the lowering of the specific activity of the methionine within the animal by admixture with the unlabeled methionine used in attempting to relieve the inhibition.

It is seen from the schedule that the labeling of the animal protein was allowed to proceed for 2.58 hours. In some experiments the time was increased to 3 hours. The total dose of ethionine, given over a period of 3 hours, was 150 mg. (0.92 mm).

When labeled glycine was given, tracer doses, similar to those used in the methionine experiments, were employed.

Mice—The experiments with mice were carried out in essentially the same manner as those with rats except that the time schedule was slightly modified and the dose of ethionine was greatly reduced. A total of 25 mg. (0.15 mm) of ethionine was given in two equal doses at 0 and 2 hours. Labeled glycine was given 1 hour following the first dose of ethionine, and the animals were sacrificed 4 hours thereafter.

These experiments were made of as short duration as possible in order to obviate any difficulty which might arise due to the deposition of lipide in the liver. This occurs only after a longer period of ethionine action (6).

Treatment of Tissues—Radioactivity not incorporated into the tissue proteins was removed as follows: The tissues were denatured by dropping them into boiling 1 M acetate buffer at pH 4.5, homogenized in glass, and washed four times with the buffer. The homogenizer was used to redisperse the preparations between each washing. With methionine as the labeled amino acid there followed 18 hours of hydrolysis with 8 N hydrochloric acid. The acid was evaporated off and the cystine sulfur separated by precipitating with cuprous oxide by the method of Zittle and O'Dell (7). The mercaptide precipitate was discarded, but the supernatant was digested with Pirie's reagent to convert the methionine sulfur to sulfate. The latter was precipitated with benzdine and filtered onto Whatman No. 1 filter paper, and its radioactivity and titratable equivalent determined.

When the radioactivity in the free cystine was determined, this was done by a mercaptide precipitation of the cystine in the first acetate buffer centrifugate with use of 6 μ M of carrier cystine (with methionine carrier also present) to facilitate the process. The mercaptide precipitate was freed of methionine contamination by reprecipitating twice in the presence of carrier methionine. Both the sulfur and radioactivity were determined as in the preceding case. The total activity in the cystine fraction was calculated from the recovered cystine sulfur, assuming an insignificant amount of free cystine in the tissue.

When glycine was used as the labeled amino acid the tissue was washed as described, then additional washings were carried out with 95 per cent alcohol (twice), hot alcohol-ether (3:1) (twice), and with ether (once) in order to remove any labeled lipide (8). The dry preparation was then homogenized in petroleum ether and sedimented onto a tared aluminum disk. A second weighing after drying off the petroleum ether gave the weight of crude protein. Radioactivity determinations were made directly on this protein preparation. In some experiments, between the washing with buffer and with the organic solvents, a wash with hot trichloroacetic acid was also used in order to remove any labeled nucleic acids (8). However, this form of contamination appears to be rather low, that is with respect to radioactivity.

Radioactivity determinations were made with use of a Tracerlab autoscaler and bell type Geiger-Müller tubes with thin mica windows and fillings at atmospheric pressure. The counting time was such as to reduce the error to less than 5 per cent, and for most samples to about 3 per cent. The observed radioactivity was corrected for decay (of the sulfur) and for self-absorption when necessary.

Results

The results are expressed in terms of specific activity of the protein-bound methionine (SA^M) in which methionine- S^{35} was used, or as specific activity of the protein preparation (SA^G) in which glycine- $C^{14}OOH$ was employed. The specific activities are defined as follows:

$$\text{Specific activity of methionine} = \frac{C}{\mu M \text{ of methionine sulfur}}$$

$$\text{Specific activity of glycine} = \frac{C}{\text{mg. of protein preparation}}$$

where C = counts per minute in sample. The values given for the specific activities in Tables I to III are comparable only within experiments, for different amounts of radioactivity were used and some variations in tube performance occurred. *All experiments are internally controlled.*

The results of a typical experiment are shown in detail in Table I. Table I shows that, although the incorporation of methionine into the proteins of individual rats shows significant differences, yet the inhibition of the incorporation by ethionine far exceeds such experimental variations. In the different experiments inhibitions of 30 to more than 50 per cent are observed in the incorporation of methionine into liver protein. Smaller inhibitions are seen in the uptake by the kidneys. This may be attributed either to the inability of kidney to take up ethionine as well as the liver or to the more rapid breakdown of ethionine in kidney tissue, if no more specific differences in protein synthesis in liver and kidney exist. Although Shen and Lewis (9) have observed the metabolic inertness of ethionine, the formation of keto acid was noted. Thus ethionine may be more rapidly removed from kidney tissue by oxidative deamination and also by excretion into the urine. It is also seen from Table I that the uptake observed in the kidney is somewhat greater than that in the liver, confirming previous observations.

In Experiment 2a the effect of oral sucrose on the uptake is noted. Apparently there is a slight stimulation by this substance (see Experiment 2, controls) although there is no relief of the inhibition by ethionine. This is to be compared with the effect of oral sucrose on the accumulation of lipide in the liver which occurs during a 12 hour period of ethionine administration (6). Sucrose, in this case, relieves the condition.

Table II shows, in summary form, that an equimolar dose of methionine when given simultaneously with the ethionine prevents the appearance of an inhibition of methionine incorporation. This inhibition ratio of unity should not be taken to be significant because nothing is known either of the behavior of the ratio of ethionine to methionine intracellularly, or of

TABLE I

Inhibition by Ethionine of Uptake of Methionine by Proteins of Female Rats

Protein	Experiment No.	Unlabeled substances*	Specific activity of proteins, counts per min. per μ M				Inhibition
			Rat 1	Rat 2	Rat 3	Average	
		<i>mM</i>					<i>per cent</i>
Kidney	1	Controls	146	165	158	$156 \pm 5.6^\dagger$	10
		0.92 ethionine	143	153	123	140 ± 8.9	
	2	Controls	68	64	53	62 ± 4.3	22
		0.92 ethionine	41		54	48 ± 6.4	
Liver	1	Controls	131	108	122	120 ± 6.7	28
		0.92 ethionine	78	90	91	86 ± 3.9	
	2	Controls	67		66	67 ± 0.5	53
		0.92 ethionine	28	29	37	31 ± 3.0	
	2a	Controls + 7.3 sucrose	70	76		73 ± 3.0	45
		0.92 ethionine + 7.3 sucrose	43	36	41	40 ± 2.1	

* In all experiments a tracer dose of labeled methionine was injected. All substances were given intraperitoneally except the sucrose in Experiment 2a. The experimental results are not intercomparable (see the text).

† All errors are standard errors of the mean.

TABLE II

Effect of Ethionine and of Ethionine Plus Methionine on Uptake of Methionine by Liver Proteins of Rats

Experiment No.	Methionine injected*	Specific activity of proteins after,† counts per min. per μ M		Inhibition
		Methionine	Methionine + ethionine‡	
	<i>mM</i>			<i>per cent</i>
1	0	120	86	28
2	0	67	31	53
2a*	0	73	40	45
3	0.92	213	200	6
4	0.92	59	63	-6
5	1.84	59	54	9
6	0.92	77	64	17
7	0.92 (L)	107	103	5

* See the foot-note to Table I.

† The values given are the averages obtained from three rats (female).

‡ In all experiments 0.92 mM (150 mg.) of ethionine was given in a divided dose (see the text for details).

TABLE III

Effect of Ethionine and of Ethionine Plus Methionine on Uptake of Glycine by Liver Proteins of Rats and Mice

Experiment No.	Specific activity of proteins after, counts per min. per mg. protein				Inhibition
	Glycine*	Glycine + methionine†	Glycine + ethionine	Glycine + ethionine + methionine	
					<i>per cent</i>
1	52.5		31.6		40
2		10.1		10.2	-1
3	44.4		29.7		33
4	55.1		45.9		17
		54.3		63.5	-17
5	33.8		23.9		29
		36.6		40.1	-10
DL-Valine instead of methionine.....				24.4	28

* Glycine in tracer doses only (all experiments).

† Methionine, 0.92 mm in Experiments 1 and 2 (rats), 0.15 mm in Experiment 4 (mice), and 0.31 mm in Experiment 5 (mice). Ethionine was given in amounts equimolar to the methionine. In Experiment 5, last line, an equimolar amount of DL-valine is used to replace the methionine.

TABLE IV

Effect of Ethionine and of Ethionine Plus Methionine on Conversion of Methionine Sulfur to Cystine Sulfur in Rats

Experiment No.	Free cystine in tissue after, counts per min.		Inhibition
	Methionine*	Methionine + ethionine†	
			<i>per cent</i>
1	75	34	55
2‡	21	5	76
3	93	59	36
4	18	12	33
5	17	14	17
6	32 (L)	27	17

* In Experiments 1 and 2 only a tracer dose of methionine was used, but in Experiments 3, 4, and 6, 0.92 mm, and in Experiment 5, 1.84 mm of methionine were used in addition to the tracer dose. In Experiment 6, the inactive methionine was the L isomer.

† The dose of ethionine used in all cases was 0.92 mm.

‡ Sucrose (7.3 mm) was orally administered to the animals in this series.

the behavior of this ratio during the progress of the experiment. Different ratios are found in tissue slices and in particulate preparations.¹

It appears also that DL-methionine is about as effective as L-methionine in these experiments. This may be attributed to the rapid inversion of the unnatural isomer in the intact animal.

The effects of ethionine on the uptake of glycine by the liver proteins of intact rats and mice are shown in Table III, from which it is seen that, surprisingly enough, the uptake of glycine is also inhibited by ethionine. Furthermore, this inhibition is prevented by equimolar amounts of methionine but not by another amino acid (valine) unrelated in structure to the ethionine. Not only does the inhibition fail to occur in the presence of both methionine and ethionine, but there is an apparent stimulation of glycine uptake.

In Table IV it is shown that the conversion of methionine to cystine is also inhibited by ethionine. The observed inhibition here is greater than the inhibition of methionine incorporation and complete reversals of the effect were never attained with the amounts of methionine used. In other words the inhibition ratio is different for this process and for the incorporation process.

DISCUSSION

In view of the fact that the inhibition of methionine uptake by ethionine is relieved by methionine, the preliminary assumption may be made that competitive inhibition has occurred. However, quite evidently the phenomenon is not an entirely simple one; otherwise ethionine would not affect the uptake of glycine. It might be thought that the uptake of glycine would be affected only in so far as the glycine taken up was bound in peptide linkages directly to methionine. Were such the case, large inhibitions would not be anticipated unless, in the proteins under investigation, methionine occurred very largely bound to glycine. With regard to the inhibition of methionine uptake several possibilities should be considered. First, ethionine may be deethylated and thence converted to methionine with a resultant dilution of the labeling agent. This would cause an apparent lowering of the specific activity of the incorporated methionine. This appears to be improbable in the light of the results obtained by Shen and Lewis (9) which have already been quoted. Ethionine is apparently very resistant to deethylation.

Second, ethionine may be incorporated into the proteins or peptides to form abnormal products which might block the metabolic system at some critical point. No support is lent to this assumption by the normal con-

¹ Simpson, M. V., and Lee, N., unpublished data.

stancy of the amino acid composition of proteins, although Csonka, Denton, and Ringel (10) have shown that the cystine and methionine contents of hen eggs may be changed by dietary means, and that the change in composition is due, at least in part, to change in composition of two individual proteins. The possibility of introducing ethionine into the protein appears to us to be of such significance that the problem is currently being investigated with ethionine-C¹⁴-ethyl.

Last, ethionine may interfere directly with lipide metabolism (6) and thus indirectly may affect the synthesis of protein. However, the results with sucrose feeding indicate that these two effects are independent. At any rate there does not appear to be any necessity for assuming such a relation, and any connection that there may be is probably too remote to result in the large changes that are observed.

In view of these various possibilities it does not appear to us to be profitable to speculate further on the nature of this inhibition. Experimental data concerning the nature of the inhibiting process can be obtained more easily with tissue slices or particulate preparations. However, it may be permissible to remark further on the inhibition of glycine uptake by methionine. The incorporation of amino acids into protein may be the result of either one of two chief types of processes. Either there may be complete *de novo* synthesis of a protein molecule from amino acids or else there may be an opening of one or two peptide bonds to release an amino acid followed by the reincorporation of the same or another similar molecule. It follows from the former hypothesis that amino acids would be entirely dependent upon one another in their formation of protein; therefore, the effective absence (by competitive inhibition, for example) of one amino acid would result in the inhibition of uptake of all the others. On the contrary, the mechanism of "opening and closing of bonds" implies that amino acids may interchange between protein and pool more or less independently of one another. Since ethionine inhibits the uptake of both methionine and glycine, and to about the same extent, it appears, superficially, at least, that the results are more readily fitted into the first picture than into the second.

The experiments support the hypothesis that the inhibitions in growth of microorganisms observed in the presence of ethionine are due, at least in part, to the inhibition of protein synthesis. However, it is possible that ethionine has other effects on the metabolism of microorganisms. Here, as in rats, the substance may also inhibit the conversion of methionine to cystine. Ethionine may inhibit the demethylation process.² It is also possible that ethionine may have some non-specific toxic effect on biological

² This is supported by results obtained with methionine-C¹⁴-methyl in experiments of a more direct nature.

systems, but since such effects are difficult to characterize, they will not be discussed.

Our best thanks are due to United States Industrial Chemicals for supplies of ethionine and methionine, and to the staff of the Crocker Laboratory, University of California, Berkeley, for very generously providing the Geiger-Müller tubes.

We are indebted to the Oak Ridge National Laboratories for the radioactive sulfur.

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SUMMARY

1. Ethionine inhibits the incorporation of methionine into the proteins of intact rats.
2. Ethionine likewise inhibits the incorporation of glycine into the proteins of intact rats and mice.
3. Methionine counteracts both these inhibitions.
4. Ethionine inhibits the conversion of methionine sulfur to cystine sulfur.
5. The results are discussed from the point of view of the inhibition of the growth of microorganisms and also from the point of view of protein synthesis.

BIBLIOGRAPHY

1. Dyer, H. M., *J. Biol. Chem.*, **124**, 519 (1938).
2. Harris, J. S., and Kohn, H. I., *J. Pharmacol. and Exp. Therap.*, **73**, 383 (1941).
3. Roblin, R. O., Jr., Lampen, J. O., English, J. P., Cole, Q. P., and Vaughan, J. R., Jr., *J. Am. Chem. Soc.*, **67**, 290 (1945).
4. McKee, R. W., and Geiman, Q. M., *Federation Proc.*, **7**, 172 (1948).
5. Tarver, H., and Schmidt, C. L. A., *J. Biol. Chem.*, **146**, 69 (1942).
6. Farber, E., Simpson, M. V., and Tarver, H., *J. Biol. Chem.*, **182**, 91 (1950).
7. Zittle, C. A., and O'Dell, R. A., *J. Biol. Chem.*, **139**, 753 (1941).
8. Winnick, T., Peterson, E. A., and Greenberg, D. M., *Arch. Biochem.*, **21**, 235 (1949).
9. Shen, C.-W., and Lewis, H. B., *J. Biol. Chem.*, **165**, 115 (1946).
10. Csonka, F. A., Denton, C. A., and Ringel, S. J., *J. Biol. Chem.*, **169**, 259 (1947).
Csonka, F. A., *Federation Proc.*, **7**, 151 (1948).

STUDIES ON ETHIONINE

II. THE INTERFERENCE WITH LIPIDE METABOLISM*

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In Paper I (1), it has been shown that ethionine depresses the incorporation of both methionine and glycine into liver proteins and also inhibits the conversion of methionine sulfur to cystine sulfur. The inhibition of these processes is prevented by methionine, but the amount required is different in each case. The present paper describes the production of fatty livers in rats by means of ethionine, together with some attempts to prevent and cure the condition.

In two published reports on the effect of ethionine on rats, Dyer (2) found accelerated loss of weight and subsequent death with no gross lesions at autopsy, while Hardwick and Winzler (3) reported a lipotropic action of ethionine. In both of these investigations ethionine was incorporated in the diet, but in the experiments reported here, ethionine was administered intraperitoneally to *fasted* female rats. Under these conditions it has been found that this analogue of methionine produces a moderate rise in the lipide content of the liver within 12 hours and a more marked rise in 24 to 48 hours. The appearance of fatty livers can be completely prevented by small doses of DL- or L-methionine.

The evidence obtained from experiments on dogs and rats, which has been reviewed by Chaikoff and Entenman (4), du Vigneaud (5), and Best and Lucas (6), indicates that methionine and choline are interchangeable as far as their lipotropic activity is concerned. The former is assumed to act in this capacity as a labile methyl donor for the synthesis of the latter. It, therefore, became of importance to determine whether choline would prevent the rise in liver lipide caused by ethionine. Under the conditions used, it has been found to be ineffective. Also, in order to determine whether methionine is specific in its capacity to prevent the fatty liver, a number of other amino acids were tested, with essentially negative results.

Miller, Ross, and Whipple (7) have shown that methionine, administered before or within 4 hours after chloroform anesthesia in dogs, protects the animals against liver necrosis and death. The possibility of a similar

* This work was done under a Fellowship of the American Cancer Society.

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ethionine effect was considered. Although the results are not conclusive, some evidence for a specific antagonism of methionine by ethionine is presented and discussed.

EXPERIMENTAL

Animals—Virgin, female, Long-Evans rats, 160 to 200 gm. in weight, were used throughout. In each individual experiment, groups of three rats were used for the control and for the experimental series. Prior to the experiment the animals were fed the stock diet.¹ Food was removed 12 hours before the first administration of the compounds tested. Water was available at all times.

Compounds—The compounds were administered intraperitoneally except when solubility, pH, or undesirable pharmacological properties precluded this procedure. In the latter case the compounds were given by stomach tube. The ethionine² used was the same as that described in Paper I (1). In the early experiments the aqueous solutions of ethionine (25 mg. per ml.) and other compounds were administered as such, while in the later experiments they were neutralized with NaHCO_3 . No difference in results was noted between the neutralized and unneutralized solutions.

Analytical Methods—For fat analyses the animals were killed by stunning and the livers were removed, weighed, and prepared for extraction, either immediately or within 12 hours. In the latter case the livers were kept frozen. The livers were ground in a mortar with sufficient anhydrous sodium sulfate to make a dry powder and were then continuously extracted with chloroform for 48 hours (8). After evaporation of the chloroform the extracted material was weighed. This material is hereafter called lipide.

Results

Production of Fatty Liver—The first experiments indicating the ability of ethionine to produce a fatty liver were performed on mice. However, since in this species starvation alone results in a marked rise in the lipide content of the liver (9), rats were used in subsequent work. While a dose of 50 mg. of ethionine produced a questionable increase in rat liver fat within 12 hours, 200 mg. (50 mg. every 2.5 hours for four doses) produced

¹ The stock diet was composed of the following (gm. per 100 gm. of diet): fish-meal 16.5, skim milk powder 13.1, alfalfa meal 8.2, ground whole corn 24.7, ground whole wheat 28.8, hydrogenated vegetable oil (Primex) 2.1, wheat germ 4.1, CaCO_3 0.4, NaCl 0.4, dried yeast 1.7, and sardine oil 0.36.

² We would like to express our thanks to United States Industrial Chemicals, Inc., for a generous gift of ethionine.

a moderate but definite rise within the same period. Table I shows that the liver lipid concentration rises to about 13 per cent under such a regimen. The results of experiments of longer duration are recorded in Table II, from which it is seen that the lipid increases to approximately 18 per cent in 36 to 50 hours. In these animals the lipid increase reached its maximal value in the 36 hour period. There was no significantly greater amount in the livers of animals sacrificed after 50 hours. Animals treated with ethionine alone do not live longer than this.

TABLE I

Effect of Some Amino Acids on Fatty Liver Produced by Ethionine

Rats sacrificed 12 hours after starting the treatment.

No. of rats*	Test substance†	Dose	Total lipid‡
		mm	per cent
21	None, no ethionine		6.1 ± 0.3
22	" with ethionine		13.4 ± 0.5
3	DL-Methionine	1.23	6.7 ± 0.4
6	"	1.23	6.6 ± 0.9
3	L-Methionine	0.63	6.6 ± 0.8
6	DL-Methionine	0.31	7.7 ± 0.6
9	L-Lysine	1.23	11.8 ± 0.8
3	Glycine	1.23	11.9 ± 0.5
9	DL-Valine	2.46	10.8 ± 0.7
3	L-Valine	0.62	9.6 ± 0.5
3	L-Alanine	0.62	11.6 ± 0.9
3	DL-Homocysteine	1.23	12.8 ± 0.5

* 170 to 200 gm., female, fasted 12 hours prior to treatment.

† All animals, except the twenty-one normal controls, received 1.23 mm of ethionine administered intraperitoneally in four equal doses at 2.5 hour intervals. Among the normal controls are included animals which were given the amino acids methionine, lysine, glycine, valine, and alanine. The liver lipid content of these animals was no different from that of animals which received water only. The test substance was given simultaneously with the ethionine.

‡ The variabilities given in this and subsequent tables are standard errors of the mean. The per cent lipid given is on the wet weight basis.

Effect of Methionine and Other Amino Acids Used Preventatively—As seen from Table I the simultaneous administration of DL- or L-methionine and ethionine, both dissolved in the same solution, completely prevents the rise in liver lipid which occurs in 12 hours. An amount of methionine, chemically equivalent to one-quarter of the dose of ethionine, is completely effective. L-Lysine, glycine, DL-valine, L-alanine, and DL-homocysteine had slight or no effect on the fat accumulation in the liver.

Since in the 12 hour experiments valine appeared to be more effective

than the other amino acids tested, it became of interest to determine the effect of this amino acid in experiments of longer duration. Table II shows that the rise in liver fat is quite marked with valine. Methionine under these conditions, however, did not completely prevent the rise. It is possible that methionine and valine are more rapidly metabolized than is ethionine. Under such conditions the ethionine might remain "unneutralized" after 24 hours. To test this hypothesis a group of animals was given, in addition to the previous treatment, small amounts of methionine or valine every 12 hours until the time of sacrifice. The results with this

TABLE II

Effect of Some Amino Acids on Fatty Liver Produced by Ethionine

Rats sacrificed 36 to 55 hours after starting the treatment.

No. of rats*	Test substance†	Dose		Character of lipide extracted	Gross appearance of adrenal
		mm	per cent		
3	None, no ethionine		6.8 ± 1.1	Solid	Normal
6	" with ethionine		18.2 ± 1.2		
1	Methionine	0.62	11.0	Solid	Normal
2	"	1.23	10.2 ± 0.2	"	"
3	"	3.71	6.2 ± 0.1	"	"
2	Valine	1.23	19.6 ± 0.9	Liquid	Hemorrhagic
2	"	3.71	16.4 ± 1.5	"	"
2	Cysteine	1.23	19.0 ± 4.0	"	"

* See Table I.

† All animals except the three normal controls (first line) received 1.23 mm of ethionine and the test substance simultaneously. Those animals receiving more than 1.23 mm of the test substance were given 0.62 mm at 12 hour intervals following the first 1.23 mm. All substances were the DL isomers (except the cysteine).

group, which received a total dose of 3.71 mm of the amino acid, are also given in Table II. It can be seen that, whereas the animals treated with 3.71 mm of methionine showed no increase in liver lipide, those treated with inadequate amounts of methionine or with other amino acids had fatty livers. These results indicate that, of the amino acids tested, methionine is the only one effective in preventing the rise in liver fat. In the preceding paper (1), it is shown that valine is ineffective in preventing the suppression by ethionine of the incorporation of labeled glycine into liver proteins in mice.

Effect of Choline and Other Lipotropic Agents—Since it was at first considered that ethionine might be producing the fatty liver through interference with transmethylation, thereby decreasing the synthesis of choline, it became of importance to test the effect of the latter under various con-

ditions. Choline, as the chloride, was administered by stomach tube, usually after neutralization with NaHCO_3 , except in Experiment 8 in which it was administered intraperitoneally. As seen from Table III, at no time did the fat content return to normal under the influence of choline. Even when, as in Experiment 5, choline chloride was incorporated into the stock diet at a level of 200 mg. per 100 gm. of diet for a period of 10 days prior to the administration of the ethionine or ethionine plus choline, no apparent effect was evident. These results show that choline is in-

TABLE III

*Effect of Some Lipotropic Agents in Prevention of Fatty Liver Produced by Ethionine**
Rats sacrificed 12 hours after starting the treatment.

Experiment No.	No. of rats†	Test substance‡	Dose	Total lipide
			mM	per cent
1-4§	11	Choline	0.31-1.43	12.3 ± 0.5
5a	3	None		14.7 ± 1.5
5b§	3	Choline	0.87	13.0 ± 1.5
7	3	<i>i</i> -Inositol	0.28	12.6 ± 1.4
7	3	Dimethylthetin	1.23	10.7 ± 0.9
8 ^c	3	DL-Homocystine	0.96	
		Choline	1.23	11.1 ± 0.1
8	3	DL-Homocystine	0.96	
		Dimethylthetin	1.23	9.0 ± 1.0

* For controls see Table I.

† See Table I.

‡ All animals received ethionine as before.

§ Choline as the neutralized chloride was administered in divided doses by stomach tube.

|| Choline (0.2 per cent) was added to the stock diet for 10 days preceding the experiment.

^c Choline was administered intraperitoneally simultaneously with the ethionine.

capable, under the present experimental conditions, of preventing this action of ethionine.

Both inositol (10) and dimethylthetin (11) have been shown to be lipotropic under some conditions. Experiment 7, Table III, indicates that they have slight if any effect on the ethionine fatty liver. Homocystine plus dimethylthetin (Experiment 8) appears to have some effect on counteracting the action of ethionine and this combination may be more effective than homocystine plus choline. Presumably these substances are active because of their ability to form methionine.

Effect of Carbohydrate—It has long been known that carbohydrate will prevent the increase in liver lipide which occurs in starvation in some spe-

cies (12). Carbohydrate was therefore tested for its effect on the fatty liver caused by ethionine. In Table IV are recorded results obtained with both glucose and sucrose. In every animal large doses of sugar prevented the rise in liver lipide. However, glucose in amounts equimolar to the administered ethionine had only a small effect. These results, therefore, are in contrast to those obtained with equimolar doses of methionine.

Sex Difference—Although ethionine readily produces fatty livers in adult females, it is far less effective in males of comparable age or weight. With young rats under 100 gm. in weight, there appears to be much less difference between the sexes. Preliminary results indicate that, after castration, mature males readily develop a fatty liver with ethionine, while

TABLE IV

*Effect of Carbohydrate in Prevention of Fatty Liver Produced by Ethionine**
Rats sacrificed 12 hours after starting the treatment.

No. of rats	Carbohydrate† administered	Dose	Total lipide
		<i>mM</i>	<i>per cent</i>
9‡	Sucrose	14.6	6.7 ± 0.2
3‡	Glucose	13.9	6.2 ± 0.6
3§	"	1.23	10.9 ± 0.3

* For the lipide content of the liver in normal controls and in those receiving ethionine only, see Table I.

† All animals received ethionine as before.

‡ Carbohydrate administered by stomach tube simultaneously with the ethionine.

§ Glucose administered intraperitoneally with the ethionine.

castrated males with pellets of testosterone are resistant. Additional investigations along these lines are being pursued.

Other Effects of Ethionine—Ethionine, in the doses used, has invariably caused death of the animals within 30 to 50 hours after the first dose. Marked gross changes are usually found in the adrenals, which show varying degrees of both cortical and medullary hemorrhage. Methionine prevents both changes; the rats remain active and apparently normal. Carbohydrate only delays the onset of death, while the amino acids tested, aside from methionine, have no beneficial effect.

Sections of liver, both paraffin and frozen, show large amounts of lipide in the cells in ethionine-treated animals. None of the sections examined has shown any evidence of necrosis, such as one sees in chloroform toxicity. Sections of kidney show the presence of lipide in the basal portions of the cells of the convoluted tubules, with no sign of necrosis. A detailed histological study is contemplated and will be reported elsewhere.

Character of Fat—In all ethionine-treated animals except those given

methionine the liver lipide was found to be liquid, whereas that from the controls or from animals showing no rise in lipide was solid.

Cure of Fatty Liver and Prevention of Death—In view of the efficacy of methionine and glucose in preventing fatty liver, it became of importance to determine whether these substances would also cure the condition. Table V records the results of experiments in which glucose or methionine was administered 12 hours after the first dose of ethionine and every 12 hours thereafter. It is apparent that (1) *glucose in large doses causes the complete or almost complete disappearance of excess lipide from the liver*, and (2) methionine is less effective in removing the lipide from the liver.

TABLE V

*Effect of DL-Methionine and Carbohydrate on Cure of Fatty Liver Produced by Ethionine**

No. of rats	Time sacrificed	Test substance†	Dose	Liver lipide
	hrs.		mM	per cent
9†	36-72	DL-Methionine	1.88- 2.48	14.1 ± 3.6
6§	30-72	Glucose	16.6 -33.3	6.6 ± 1.0
3‡	30-50	"	2.46	14.4 ± 0.6

* For the lipide content of liver in normal controls and in those receiving ethionine only, see Table II.

† All animals received ethionine as before. The test substance was first administered 12 hours after the first dose of ethionine. The dose was repeated every 12 hours until the animal died or was sacrificed.

‡ Test substance administered intraperitoneally.

§ Test substance administered by stomach tube.

However, the latter always prevents death and the adrenal changes. The rats remain alive and healthy in contrast to those treated with glucose, which eventually die.

DISCUSSION

Under the experimental conditions reported, choline is not effective in preventing the rise in liver lipide. The evidence to date indicates that the rôle of methionine as a lipotropic agent resides in its ability to donate methyl groups for the synthesis of choline. If ethionine were producing the fatty liver by blocking the demethylation of methionine, the condition should be relieved by choline. Since this does not appear to be so, one is forced to postulate the existence of either a methionine-choline interrelationship more complex than has hitherto been suggested, or some other rôle of methionine in lipide metabolism. The absence of any significant effect from dimethylthetin serves to emphasize further the inadequacy of

the prevailing theories in explaining the production of fatty livers with ethionine.

In addition to the inefficacy of choline, the prevention and cure with large doses of glucose and the different sex susceptibility (13) appear to place this type of fatty liver in a group apart from that arising from a deficiency of choline and labile methyl groups. It is possibly more closely akin to the fatty liver caused by starvation. In some respects the inhibition of protein synthesis in the presence of ethionine may with some justification be compared to the lack of protein formation in the absence of the component amino acids.

The relationship of glucose to this type of fatty liver and to methionine is difficult to assess at this time. However, in this connection it should be emphasized that the results reported here are not in conflict with those of Hardwick and Winzler (3). They obtained a lipotropic effect when ethionine was given along with a diet containing ample carbohydrate. Under these conditions and in short term experiments, a lipide deposition in the liver would not be anticipated from our results.

It is important to establish, if possible, whether ethionine is acting as a specific antagonist of methionine or is simply a "toxic agent," to be placed in a class with chloroform, carbon tetrachloride, etc. Although the evidence is not wholly unequivocal, the following facts support the concept of a specific ethionine-methionine relationship: (1) Three different amounts of methionine on a molar basis are required to prevent the three effects investigated, *i.e.* fatty liver (at most one-fourth the molar amount of ethionine), inhibition of incorporation of labeled amino acids into liver protein *in vivo* (about equimolar), and the conversion of methionine-S to cystine-S (about twice molar). This suggests that ethionine acts at specific biochemical loci within the cell, rather than on the cell as a whole. (2) In experiments of more than 24 hours duration, methionine must be administered at intervals in order effectively to prevent the ethionine from causing a rise in liver fat (Table II). This suggests that the ability of ethionine to produce a fatty liver depends upon the amount of methionine present at any time. When the amount of the latter in the body is maintained, ethionine appears to be ineffective. (3) Methionine is effective in preventing severe illness or death due to chloroform, but is active only within a period of 4 hours following the administration of the anesthetic (6). (4) No necrosis has been seen in sections of the livers of animals treated with ethionine, in contrast with the findings in chloroform, carbon tetrachloride, or phosphorus poisoning.

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SUMMARY

1. Ethionine is capable of inducing fatty livers in fasted female rats in 12 hours.
2. Of a series of amino acids tested, methionine alone is able to counteract this effect.
3. Choline and other known lipotropic agents have little or no effect on this type of fatty liver.
4. Carbohydrate, in large doses, prevents or cures the fatty liver condition.
5. The lipide which accumulates in the liver under the influence of ethionine is liquid following extraction, whereas the normal lipide is solid.
6. Evidence is presented to indicate that ethionine is a specific antagonist of methionine.

BIBLIOGRAPHY

1. Simpson, M. V., Farber, E., and Tarver, H., *J. Biol. Chem.*, **182**, 81 (1950).
2. Dyer, H. M., *J. Biol. Chem.*, **124**: 519 (1938).
3. Hardwick, V. L., and Winzler, R. J., *Proc. Soc. Exp. Biol. and Med.*, **69**, 217 (1948).
4. Chaikoff, I. L., and Entenman, C., *Advances in Enzymol.*, **8**, 171 (1948).
5. du Vigneaud, V., *Harvey Lectures*, **38**, 39 (1942-43).
6. Best, C. H., and Lucas, C. C., in Harris, R. S., and Thimann, K. V., *Vitamins and hormones*, New York, **1**, 1 (1943).
7. Miller, L. L., Ross, J. F., and Whipple, G. H., *Am. J. Med. Sc.*, **200**, 741 (1940).
8. Channon, H. S., Platt, A. P., and Smith, J. A. B., *Biochem. J.*, **31**, 1736 (1937).
9. Barrett, H. M., Best, C. H., and Ridout, J. H., *J. Physiol.*, **93**, 367 (1936).
10. Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **139**, 485 (1941).
11. Quoted by du Vigneaud, V., Moyer, A. W., and Chandler, J. P., *J. Biol. Chem.*, **174**, 477 (1948).
12. Peters, J. P., and Van Slyke, D. D., *Quantitative clinical chemistry, Interpretations*, Baltimore, 2nd edition, **1**, 424 (1946).
13. Shipley, R. A., Chudzik, E. B., and György, P., *Arch. Biochem.*, **16**, 301 (1948).

FURTHER STUDIES ON THE METABOLISM IN VITRO OF RADIOACTIVE TRILAURIN AND SODIUM OCTANOATE*

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The *in vitro* oxidation of radioactive emulsified trilaurin ($-\text{C}^{14}\text{OO}-$) and sodium octanoate ($-\text{C}^{14}\text{OO}-$) has been reported (1). It was shown that rat tissue slices metabolized these lipides, as judged by the appearance of radioactivity in the respired carbon dioxide, and that there was a considerable difference between various tissues with respect to their ability to catabolize these materials. Studies with liver slices disclosed that in this tissue lipide oxidation continued for a very long time, first at a fairly rapid rate and then at a somewhat slower, although significant, rate. Further studies have been undertaken in which various supplementary substrates and inhibitors were added together with the labeled lipides. Malonate inhibition was especially studied, both from the standpoint of the inhibition itself and also from that of its counteraction. In this paper studies are reported in which the tissues used were liver and kidney, and in which the criterion of metabolism of the lipides was the amount of radioactivity which appeared in the respired carbon dioxide.

EXPERIMENTAL

Malonate Inhibition Studies—The rôle of the tricarboxylic acid cycle in fatty acid metabolism was determined by the use of malonic acid as an inhibitor. Approximately 100 to 200 mg. (dry weight) of liver or kidney slices were incubated in Ca-free Ringer-phosphate solution with 0.001 M octanoic acid (as Na salt) and 0.002 M malonic acid (as Na salt). A similar flask without malonate was used as the control, and a third contained both the malonate and 0.002 M fumaric acid. The incubations were carried out for 2 hours at 37.5° and a pH of 7.1 with an O_2 atmosphere. The respired CO_2 was collected and the activity determined as previously described (1). The results of a typical experiment are given in Table I, together with those obtained when 25 mg. of radioactive trilaurin

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(—C¹⁴OO—) were added in the form of an emulsion (1) to each flask in place of the octanoic acid. It is evident that fatty acid oxidation is inhibited by malonic acid and that fumaric acid does not overcome this inhibition. Two samples each of malonic and fumaric acids obtained from different sources were used with similar results, and neutralization equivalents on each acid were within 0.2 per cent of the theoretical value.

TABLE I

Effect of Malonate and Fumarate on Oxidation of Octanoate and Trilaurin by Rat Liver and Kidney Slices

Radioactive substrate	Tissue	Flask No.*	Supplements		Dry tissue	Total c.p.m. in CO ₂ per gm. dry tissue	Specific activity†
			Malonate	Fumarate			
			M	M	mm CO ₂ per gm.		
Emulsified trilaurin‡	Liver	1			0.527	1,441	227
		2	0.005		0.518	724	116
		3		0.005	0.765	1,688	184
		4	0.005	0.005	0.694	1,043	125
Emulsified trilaurin‡	Kidney	1			0.815	5,790	594
		2	0.005		0.795	2,750	291
		3		0.005	1.03	4,740	383
		4	0.005	0.005	0.735	2,260	257
Octanoic acid§	Liver	1			0.512	4,210	685
		2	0.005		0.421	1,660	328
		3		0.005	0.780	5,320	569
		4	0.005	0.005	0.570	2,910	426
“ “	Kidney	1			0.853	83,300	8160
		2	0.005		0.706	36,600	4300
		3		0.005	1.565	101,200	5400
		4	0.005	0.005	1.080	65,300	5020

* All the flasks contained 20 ml. of Ca-free Ringer-phosphate solution (pH 7.1) and approximately 200 mg. of liver or 100 mg. of kidney (dry weight); incubation time, 2 hours.

† Counts per minute per mg. of carbon in CO₂.

‡ 25 mg. of trilaurin (—C¹⁴OO—) as an emulsion; activity, 920 counts per minute per mg. of trilaurin.

§ 0.93 mg. and 9.3 mg. of octanoic acid (—C¹⁴OO—) added to liver and kidney respectively. Activity, 6500 counts per minute per mg. of acid.

To determine whether an excess of fumaric acid would be effective in counteracting the malonate inhibition, various amounts of malonate were added to a series of flasks which contained trilaurin, Ca-free Ringer's solution, 0.020 M, fumaric acid, and approximately 100 mg. of liver slices (dry weight). The exact amounts of materials added to each flask are given in the foot-note to Table II, which gives the results of a typical experiment.

The data show that inhibition occurs even when there is a preponderance of fumaric acid.

Effect of Various Supplements on Fatty Acid Oxidation and on Malonate

TABLE II

Malonate Inhibition of Trilaurin Oxidation in Presence of Fumarate

Flask No.*	Malonate concentration	Fumarate concentration	C.p.m. in CO ₂ per gm. liver (dry weight)	Specific activity
	M	M		
1	0.0000	0.0140	1030	640
2	0.0024	0.0140	705	565
3	0.0070	0.0140	410	325
4	0.0140	0.0140	360	260

* All the flasks contained 20 ml. of Ca-free Ringer-phosphate solution (pH 7.1), 25 mg. of trilaurin ($-C^{14}OO-$) as an emulsion (0.5 ml.), approximately 500 mg. of liver slices (dry weight), and malonate and fumarate at the concentrations indicated; total activity per flask, 23,000 counts per minute; incubation time 2 hours.

TABLE III

*Relative Effects of Various Supplements on Fatty Acid Oxidation and on Malonate Inhibition**

Supplement,† 0.005 M	Total CO ₂ respired per gm. dry tissue		Total activity in CO ₂ per gm. dry tissue		Specific activity of CO ₂	
	No malonate	Malonate, 0.005 M	No malonate	Malonate 0.005 M	No malonate	Malonate, 0.005 M
None.....	100	82	100	48	100	59
Fumarate.....	152	112	126	65	83	58
Malate.....	136	115	129	66	95	57
Oxalacetate.....	174	144	125	52	72	50
α -Ketoglutarate.....	158	133	123	64	78	48
Pyruvate.....	127	116	123	54	97	46

* Table III is a composite of typical results from numerous smaller experiments. To facilitate comparison between the effects of the various supplements, the values for the control used in each experiment have been arbitrarily designated as 100, and all other values are relative to this.

† All the flasks contained 20 ml. of Ca-free Ringer-phosphate solution (pH 7.1) which contained 0.0003 M octanoic acid ($-C^{14}OO-$) as its sodium salt in addition to any added supplements. The incubation time was 2 hours and approximate weight of tissue (dry) 100 mg.

Inhibition—A number of the members of the tricarboxylic acid cycle were studied to test their effect on fatty acid oxidation and also on malonate inhibition. Reports in the literature indicate that various dicarboxylic acids influence fatty acid metabolism (2-4). A number of experiments

were performed with liver slices in a manner similar to that described above. Each experiment contained a series of flasks which contained sodium octanoate, and, except for the control, also an additional substrate or malonic acid, or both. The pertinent data and relative results of typical experiments are given in Table III. It is apparent that all of the various supplements used increased the amount of radioactive carbon respired by approximately the same extent, but that none of them corrected the malonate inhibition.

TABLE IV
*Relative Effects of Various Inhibitors on Octanoate Oxidation**

Tissue	Octanoate concentration	Inhibitor	Inhibitor concentration	Total CO ₂ respired per gm. dry tissue	Total activity in CO ₂ per gm. dry tissue	Specific activity of CO ₂
	<i>M per l.</i>		<i>M per l.</i>			
Liver	0.0003			100	100	100
"	0.0003	Malonate	0.005	74	25	34
"	0.0003	Maleate	0.005	101	69	68
"	0.0003	Dinitrophenol	0.005	73	73	100
"	0.0003	Iodoacetate	0.005	47	9.5	20
"	0.0003	" + 0.005 M pyruvate	0.005	77	9.1	11.8
"	0.0003	β -Iodopropionate	0.005	79	123	156
"	0.0003	NaF	0.005	87	120	138
"	0.0033	"	0.005	76	34	44
"	0.0003	NaN ₃	0.005	101	176	174
"	0.0033	"	0.005	98	54	52
"	0.0033	"	0.010	122	51	42
Kidney	0.0033	"	0.005	106	21	19
"	0.0033	"	0.010	97	36	37

* The data were recorded and experiments conducted as explained in Table III, except that octanoate and inhibitor concentrations were varied.

Effect of Various Inhibitors on Fatty Acid Metabolism—The following inhibitors were tested: sodium azide, iodoacetic acid, β -iodopropionic acid, maleic acid, sodium fluoride, and 2,4-dinitrophenol.¹ Some of these substances were tested at several substrate concentrations because preliminary studies indicated a "substrate concentration" effect. Several concentrations of some of the inhibitors were also tested. Pertinent data and relative results of typical experiments are given in Table IV. Inhibition occurred with iodoacetate, malonate, maleate, azide, and fluoride,

¹ Several of the experiments in this and the following section are taken from some related current studies. The assistance of Mrs. Alice Robbins and Mrs. Mary Cunningham in these latter studies is appreciated.

but the latter two actually caused a stimulation at the lowest octanoate concentrations used. The lack of an effect on the specific activity of the CO_2 when dinitrophenol was added is of interest since it uncouples phosphorylation and respiration (5). This phenomenon is being studied further.

*Effect of Malonate and Fumarate in Prolonged Incubation Experiments—*In a previous report (1) it was shown that, when emulsified trilaurin or

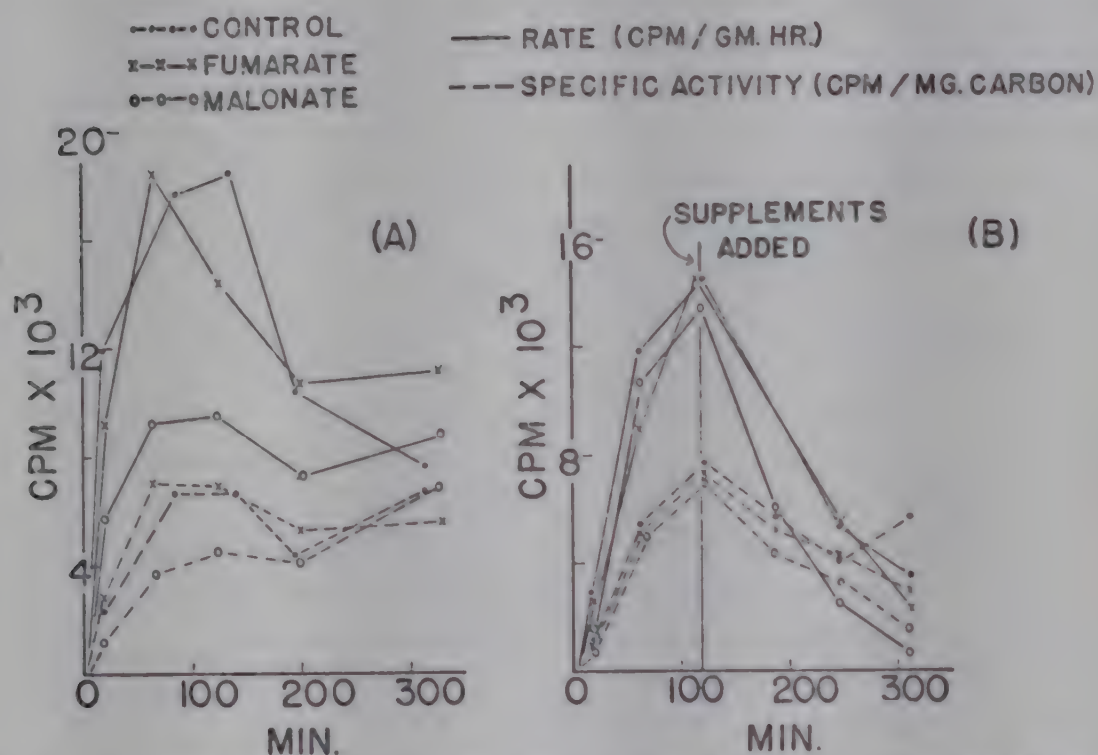


FIG. 1. Octanoate oxidation by liver slices. All the flasks contained a final total volume of 20 ml. of Ca free Ringer-phosphate solution which contained 0.0033 M of octanoic acid ($-\text{C}^{14}\text{OO}-$) as its sodium salt. The activity of the octanoic acid was 6500 counts per minute per mg. Approximately 300 mg. of liver slices (dry weight) were used in each flask and the pH was 7.1. Experiment A, 0.005 M of malonate or 0.005 M of fumarate was present in two of three flasks at zero time; Experiment B, 0.005 M of malonate or 0.005 M of fumarate was added to two of three flasks after an initial unsupplemented incubation period.

sodium octanoate was incubated with liver slices for long periods of time, a two-step curve resulted when the respired activity of successive serial samples of CO_2 was plotted against time. The effects of malonate and fumarate on this phenomenon were determined by incubating approximately 200 mg. of liver slices (dry weight) with 0.0033 M octanoic acid and with and without malonic acid or 0.005 M fumaric acid present from the beginning. Successive samples of respired CO_2 were collected and assayed for activity. In a second series of similar experiments, the malonate or fumarate was added after 120 minutes without interrupting the incubations. Typical experimental data and results of each type of experiment

are given in Fig. 1. It is apparent that fumarate had little effect on the type of curve obtained, but that malonate almost entirely destroyed the initial rapid phase of the metabolism. The slope of the curve obtained with the latter compound resembles that of the second, or slower, phase of the control curve.

DISCUSSION

Fatty acid oxidation by liver and kidney slices is strongly inhibited by malonic acid, but the inhibition is not counteracted by an equimolar amount of fumaric, malic, oxalacetic, α -ketoglutaric, or pyruvic acid, or by large amounts of fumaric acid. This phenomenon occurs in spite of the fact that each of these compounds *per se* stimulates fatty acid metabolism by approximately 20 per cent over the normal amount (see Table III). The failure to correct the inhibition by the addition of these compounds suggests that the malonic acid might act to block fatty acid metabolism prior to the tricarboxylic acid cycle or at one or more points in the cycle itself. As seen from the data in Table III, the total respired carbon dioxide increased to normal or above when these compounds were added to the malonate-inhibited system, which indicates that these compounds presumably reached the α -ketoglutarate (and perhaps the succinate) stage. Thus, if any of the reactions between fumarate and these latter stages were necessary for fatty acid metabolism, they were not able, of themselves, to overcome the inhibition. Although this would suggest that the site of the malonate action was on the fatty acid metabolism prior to the tricarboxylic acid cycle, it is possible to explain the results solely on the basis of the blocking of the succinate \rightleftharpoons fumarate reaction.

It has been shown (6) that when carboxyl-labeled acetoacetate was incubated with tissue preparations, carboxyl-labeled citric acid was found as an intermediate, presumably as the result of the Breusch-Wieland condensation. If this is the major pathway for the fragments of fatty acid oxidation to enter the tricarboxylic acid cycle, part of the original labeled carboxyl groups in the fatty acids would appear in the respired carbon dioxide prior to the succinate \rightleftharpoons fumarate reaction, and part would not. It would be necessary for the latter active groups to go from succinate through the various steps of the cycle to the oxalosuccinate or α -ketoglutarate stage before any additional part of this activity would appear in the CO_2 . If, however, malonate is present to block the active succinate from further reaction, most of its activity will not appear in the CO_2 . The addition of fumaric acid to such a system will not alter the active succinic acid, though it may increase the respired activity somewhat by furnishing more oxalacetic acid to condense with new active fatty acid fragments. This proposed possibility is summarized in Fig. 2.

The effect of substrate level on the influence of sodium azide and sodium fluoride on the respired radioactivity is of interest. As shown in Table

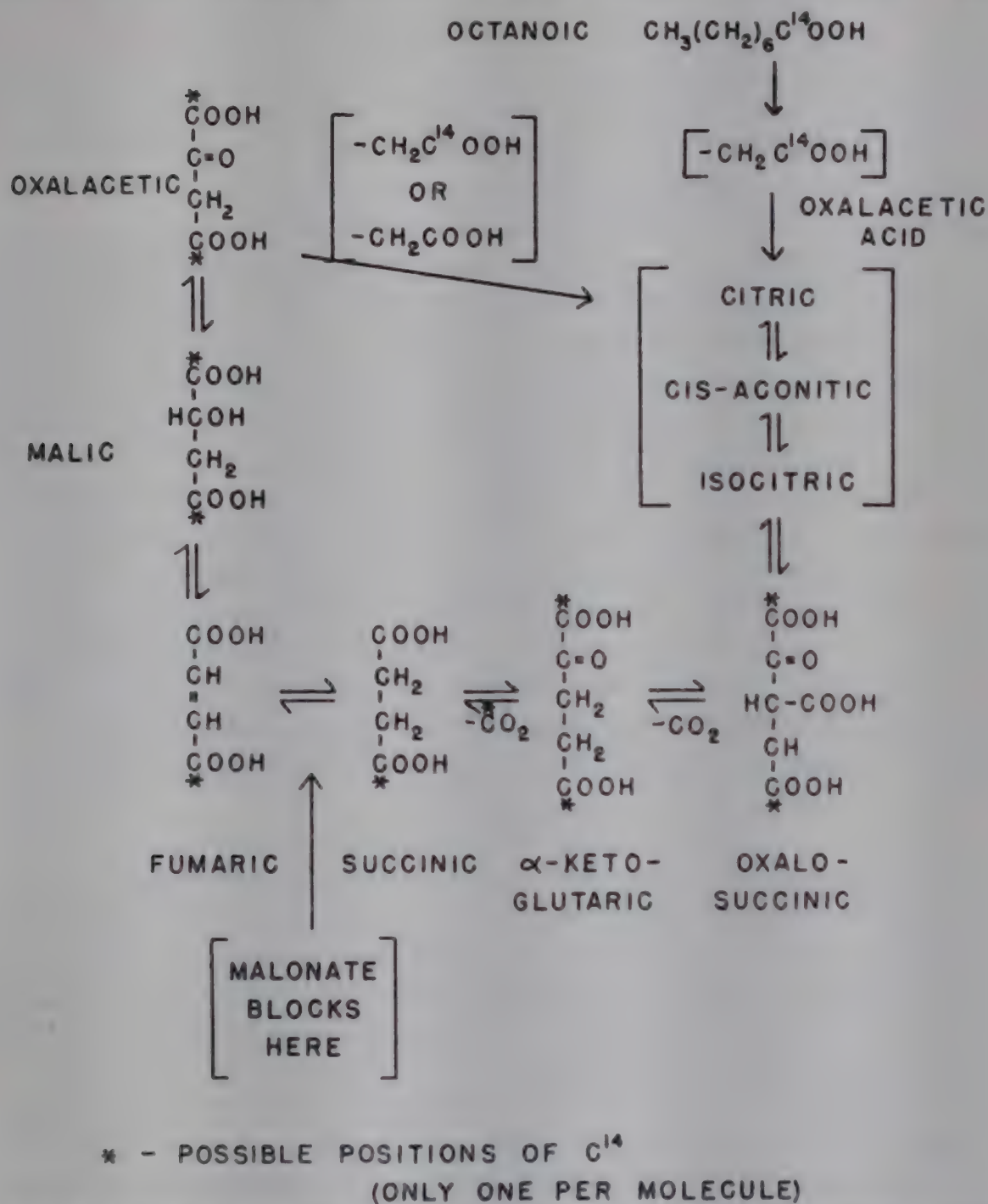


FIG. 2. Metabolic pathway of octanoate carboxyl during first passage through tricarboxylic acid cycle. The release as CO_2 of the isotopic carbon in the succinic acid can occur only when the intact cycle is operating; thus, the malonate inhibits with or without added fumarate. Additional evidence in support of this explanation will appear in a subsequent publication in which $\text{CH}_3(\text{CH}_2)_5\text{C}^{14}\text{H}_2\text{COOH}$ has been used as the substrate.

IV, the former resulted in a 72 per cent increase above normal at an octanoic acid concentration of 0.0003 M, but, when the substrate concentration

was raised to 0.0033 M, a decrease of 47 per cent from normal resulted. A similar, but smaller, effect was obtained with NaF. Thus it would appear that the effect of some inhibitors may depend to a certain extent upon the concentration of the substrate. Iodoacetic acid proved to be a strong inhibitor of the fatty acid oxidation even at substrate concentrations of 0.0003 M, and added pyruvate had no effect on the oxidation, except to increase total CO_2 . β -Iodopropionic acid was tested as an inhibitor, but resulted in slight increases in the isotopic CO_2 respired. Maleic acid depressed both total respiration and fatty acid oxidation.

The data presented in Fig. 1 show that the decreased rate of fatty acid metabolism which occurs after 120 minutes is not due to a lack of fumaric acid. It is possible, however, that one or more of the reactions in the tricarboxylic acid cycle is no longer proceeding normally and thus radioactive carbon which enters the cycle can no longer be oxidized to CO_2 as readily as before. The fact that malonate added at zero time decreased the initial fast oxidation rate, but had little effect on the later slower rate, suggests that in the unblocked system the defect which occurs in the later period lies somewhere between succinic and oxalacetic acid. In other words, the activity which appears in the CO_2 in the later stages might be primarily derived from the α -carboxyl of α -ketoglutaric acid, because the activity in the γ -carboxyl of this acid cannot be liberated by passing through the remainder of the cycle and back into oxalosuccinic and α -ketoglutaric acids from which this residual activity could then be derived.

SUMMARY

1. The amount of radioactive carbon dioxide derived from carboxyl-labeled lipides incubated with tissue slices was increased by the addition of fumarate, malate, oxalacetate, pyruvate, and α -ketoglutarate.

2. Malonate decreased the activity of the respired CO_2 in such a way that the concurrent addition of any of the above compounds had little effect on the malonate inhibition.

3. Inhibition was also shown with several other inhibitors, though in a few cases the inhibition was dependent on the substrate concentration.

4. The results are discussed and a possible explanation is presented.

BIBLIOGRAPHY

1. Geyer, R. P., Matthews, L. W., and Stare, F. J., *J. Biol. Chem.*, **180**, 1037 (1949).
2. Leloir, L. F., and Muñoz, J. M., *J. Biol. Chem.*, **153**, 53 (1944).
3. Lehninger, A. L., *J. Biol. Chem.*, **161**, 437 (1945).
4. Knox, W. E., Noyce, B. N., and Auerbach, V. H., *J. Biol. Chem.*, **176**, 117 (1948).
5. Cross, R. J., Taggart, J. V., Covo, G. A., and Green, D. E., *J. Biol. Chem.*, **177**, 655 (1949).
6. Weinhouse, S., Medes, G., and Floyd, N. F., *J. Biol. Chem.*, **166**, 691 (1946).

A MICROMODIFICATION OF THE DYE METHOD FOR THE DETERMINATION OF BASIC DRUGS IN BLOOD PLASMA

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The ability of certain acid dyes to form organic solvent-soluble complexes with basic organic compounds has been utilized by several workers for the determination of drugs in biological fluids. Lehman and Aitken (1) used brom thymol blue for the determination of demerol (1-methyl-4-phenylpiperidine-4-carboxylic acid ethyl ester hydrochloride), and later Scott and Chen (2) applied the same dye to the estimation of methadone (6-dimethylamino-4,4-diphenyl-3-heptanone hydrochloride). This drug has also been determined in urine by Cronheim and Ware (3) by means of brom cresol purple. Brodie and Udenfriend (4) and Brodie, Udenfriend, and Dill (5) applied methyl orange to the determination of cinchonidine and cinchonine.

The dye method involves equilibration of a solution of the basic drug in a suitable organic solvent with aqueous dye solution, or direct extraction of the dye-drug complex from aqueous solution into the solvent. In either case, a quantity of dye proportional to the amount of drug present passes into the solvent phase. If the organic solution is used directly for color depth comparison, or if the dye is extracted into water for macrocolorimetry, the method is frequently not sufficiently sensitive for practical application. If the dye is reextracted into a small volume of water and microcolorimetry employed, greater sensitivity may be attained. For example, as little as 0.5 γ of cinchonine has been successfully estimated (5).

There is another way, however, by which the sensitivity of the method can be increased. If a buffered solution of dye and an aqueous solution of alkali are separated from one another by a solution of drug in organic solvent, and the system is agitated in a container designed to prevent mixing of the two aqueous phases, dye will be extracted from the buffered solution and passed through the organic phase to the alkaline solution. The rate of dye transfer will be proportional to the concentration of drug in the organic phase.

On the basis of this concept a method has been devised, by means of which as little as 0.01 γ of certain basic drugs can be detected, and 0.1 γ determined with a precision of about ± 10 per cent.

EXPERIMENTAL

Apparatus

Eggerth, Littwin, and Deutsch (6) described a vessel for the continuous extraction of histamine from alkaline solution into organic solvent and thence into aqueous acid. The apparatus, two glass bulbs joined at the bottoms by a V-tube but unconnected and open to the air at the top, was

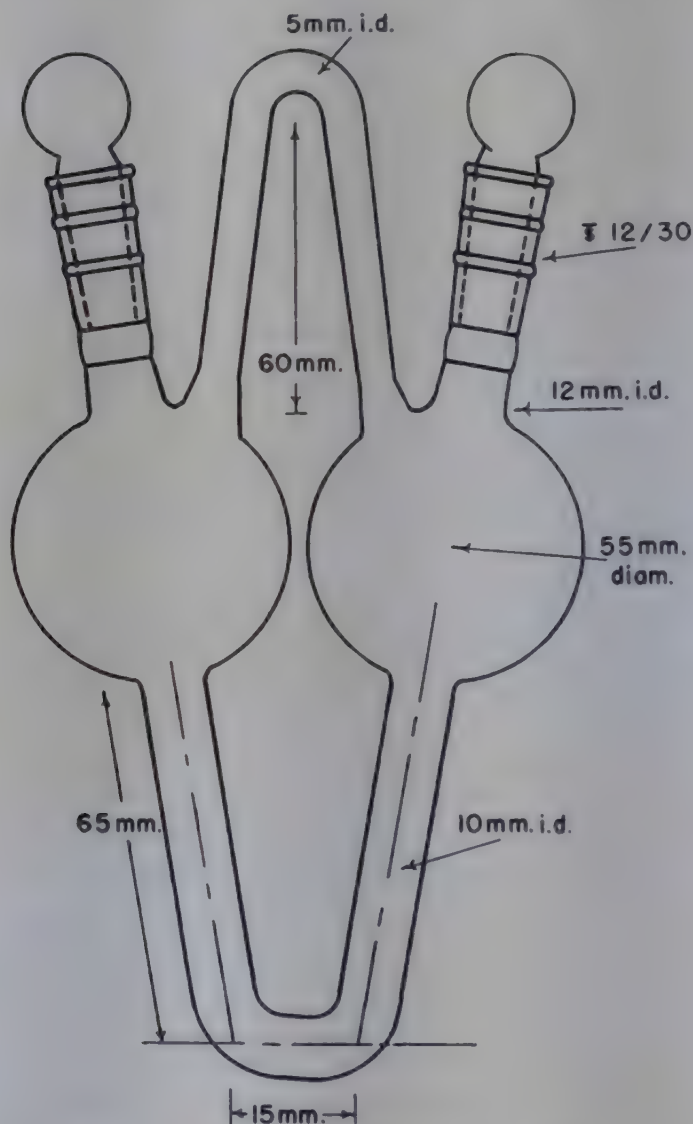


FIG. 1. Continuous extraction vessel

designed for use with solvents more dense than the aqueous solutions employed. This vessel, modified as shown to prevent air-borne contamination (Fig. 1),¹ was used in the present work. Since attempts to close the vessel with rubber tubing were unsuccessful due to contamination from the rubber, an all-glass construction was employed. The

¹ The vessels were obtained from Mr. W. R. Kump, 1062 Leesville Avenue, Avenel, New Jersey.

rocking apparatus (Fig. 2) was constructed so that the vessels were tilted about 9° to each side from the vertical nine times per minute.

Materials

Solvent—Ethylene chloride, purified (Amend Drug and Chemical Company), treated with about 15 gm. of charcoal (Darco) per liter in order to remove traces of basic materials, and filtered. Carbon tetrachloride, reagent (Merck).

The two solvents are mixed in equal proportions by volume. The mixed solvent is treated with charcoal and filtered immediately before use to guard against possible contaminating during storage.

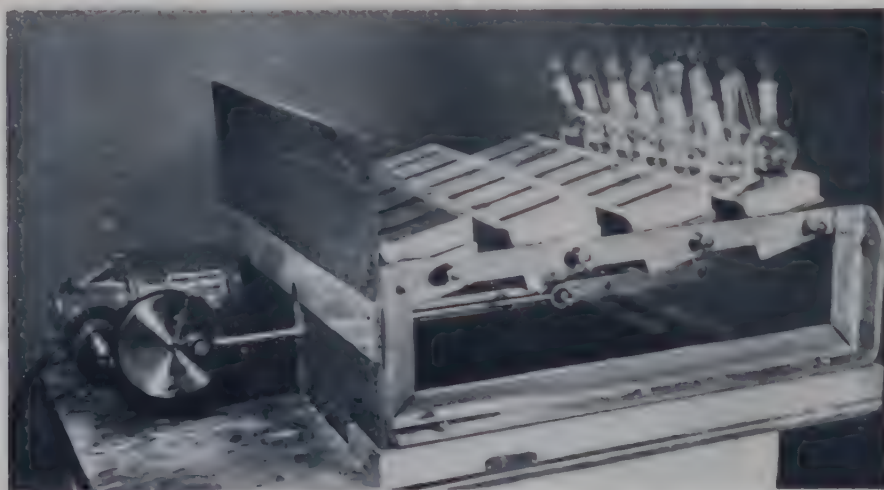


FIG. 2. Rocking apparatus for twenty-four cells

Methyl Orange—A solution containing 65 mg. of the sodium salt in 100 ml. of 0.5 M boric acid is extracted four or five times with 50 ml. of charcoal-treated ethylene chloride. The solution is diluted with an equal volume of water and shaken with mixed solvent before use.

Sodium Hydroxide—0.2 N, shaken with the mixed solvent before use.

Sodium Hydroxide—4.0 N.

Hydrochloric Acid—1:10.

Procedure

1 to 5 ml. of oxalated plasma, in a 125 ml. glass-stoppered Erlenmeyer flask, is diluted to 5 ml. with water. Following the addition of 1 ml. of 4 N NaOH and 40 to 60 ml. of mixed solvent, the mixture is shaken mechanically 20 minutes. The liquid is transferred to a centrifuge tube and the phases separated by centrifugation. 30 to 50 ml. of the organic phase are transferred to a continuous extraction vessel and diluted to 50 ml. with mixed solvent. Diluted methyl orange, 2 ml., and 0.2 N NaOH, 3.5 ml., are added to opposite bulbs of the vessel. The bulbs are stoppered firmly,

and the vessel is rocked 16 to 64 hours. 3 ml. of the alkaline solution are transferred to a test-tube and diluted to 6 ml. with 1:10 HCl. The depth of color is compared with a suitable blank at 510 m μ .²

Portions of a control plasma sample, to which have been added graded amounts of drug (0 to 1 γ), are processed simultaneously with plasma samples containing unknown quantities of drug. A reference curve is obtained by plotting optical density of the acidified methyl orange solution against drug content.

Neoantergan Concentrations in Dog Plasma—Fasted mongrel dogs, weighing 10 to 12 kilos, were given neoantergan maleate (*N*-*p*-methoxybenzyl-

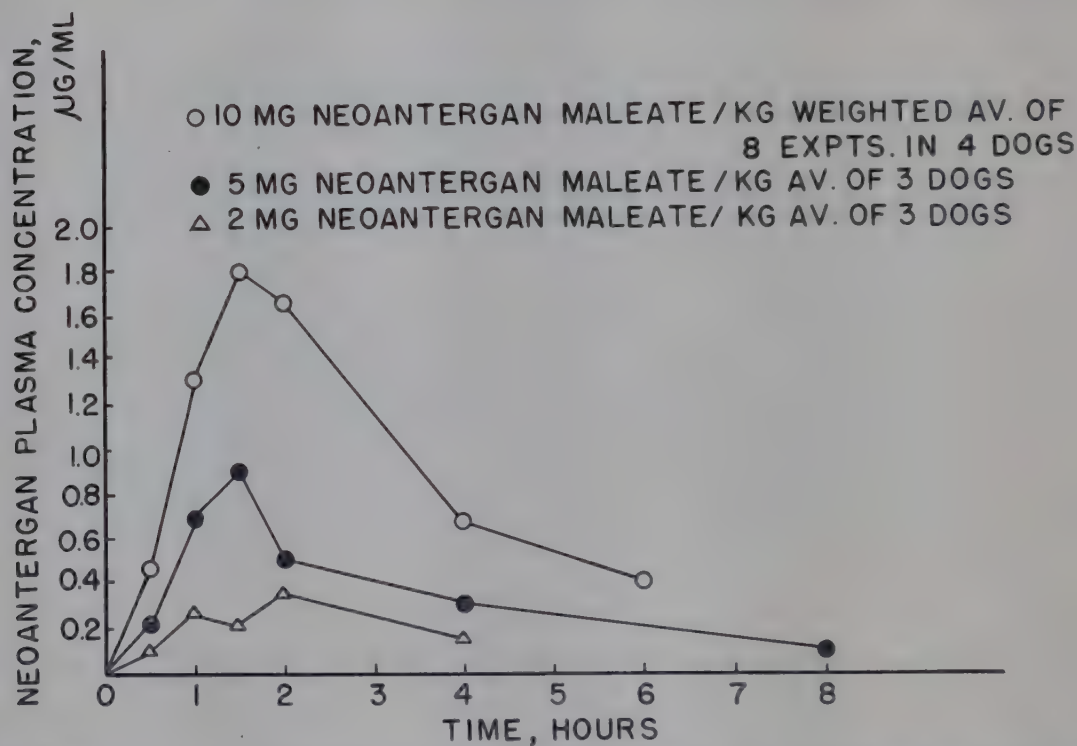


FIG. 3. Plasma concentrations of neoantergan after oral administration to dogs

N', *N*'-dimethyl-*N*- α -pyridylethylene diamine maleate) solutions by stomach tube. Blood samples were taken from the jugular vein before dosing and at intervals after dosing for 6 to 8 hours. Drug assays were made in triplicate on 1 to 4 ml. aliquots of plasma.

Fig. 3 indicates the plasma levels of drug (calculated as the maleate) after doses of 2, 5, and 10 mg. of the salt per kilo of body weight. Peak concentrations of 0.4, 0.9, and 1.8 γ per ml. of plasma were reached in 1 to 2 hours. There were no detectable quantities of neoantergan in the plasmas 12 to 24 hours after the drug had been administered.

2 to 10 per cent of the dose was found in the 24 hour urines, the lowest

² The Coleman universal spectrophotometer, model 11, with a 0.5 inch square cuvette, was used.

recovery being from the lowest dose. Although the microprocedure can be employed for the determination of drug levels in urine, the usual macro-technique was used, since great sensitivity was not required.

Characteristics of Method

Reproducibility—After a 16 hour rocking period, with 1 γ of methadone hydrochloride in each of the twenty-four cells, the average optical density of the acidified methyl orange solutions was 0.295 ± 0.002 (not corrected for blank). The maximum deviation from the mean was 7.5 per cent and the average deviation 2.6 per cent. In no experiment was the maximum

TABLE I

Standard Curves for Methadone Hydrochloride and Neoantergan Malcate

Four cells for each drug level; 16 hours rocking.

Drug level	Methadone		Neoantergan	
	Optical density* \pm S.E.	Optical density per microgram	Optical density \pm S.E.	Optical density per microgram
0	0.016 ± 0.0022		0.019 ± 0.0010	
0.1	0.037 ± 0.0016	0.210		
0.25	0.062 ± 0.0029	0.184	0.061 ± 0.0020	0.168
0.5	0.109 ± 0.0025	0.186	0.112 ± 0.0024	0.186
0.75			0.159 ± 0.0035	0.187
1.0	0.226 ± 0.0020	0.210	0.219 ± 0.0033	0.200
1.5			0.305 ± 0.0033	0.191
2.0	0.427 ± 0.0034	0.205		
Average		0.199		0.186

* The optical density ($-\log T$) in all the tables was measured in the Coleman colorimeter.

deviation from the mean greater than 7.5 per cent, but repeated experiments with 1 γ of methadone yielded average optical density values as much as 20 per cent divergent from 0.295. It is apparent that standards must be included in each assay.

No tendency was shown by individual vessels to yield high or low values, although there was some variation in dimensions among vessels.

Variation of Response with Concentration of Drug—The optical density ($-\log T$) of the acidified, transported methyl orange varies linearly with the concentration of drug in the organic phase (Table I). In these experiments with neoantergan and methadone, about 10 moles of methyl

orange were transported from the boric acid to the alkali solutions per mole of drug.

Time of Rocking—A 16 hour shaking period is convenient, but greater sensitivity can be realized with longer periods. The sensitivity obtainable with a 64 hour rocking period is illustrated by the data in Table II. In this case, 50 to 60 moles of methyl orange were transported per mole of methadone. The magnitude of the blank is a limiting factor in obtaining

TABLE II

Response to Small Quantities of Methadone Hydrochloride

64 hours rocking.

Drug level	Optical density*	Optical density per microgram
γ		
0.01	0.017	1.7
0.05	0.046	0.9
0.1	0.100	1.0

* Corrected for blank.

TABLE III

Effect of Variation in Volumes of Aqueous Phases and Solvent Composition on Response to 1.0 γ of Neoantergan Maleate

16 hours rocking.

NaOH	Methyl orange	Ratio, ethylene chloride-CCl ₄	Relative response	Optical density of blank
<i>ml.</i>	<i>ml.</i>			
3.5	2	1:1	1	0.018
3.5	4	1:1	1.1	
7.0	4	1:1	0.6	
7.0	2	1:1	0.4	
3.5	2	1:3	0.4	0.007
3.5	2	3:1	2.1	0.026
3.5	2	1:0	2.5	0.130

greater sensitivity. After shaking 64 hours, the blanks had an optical density of about 0.130, when read in the 0.5 inch cell in the Coleman colorimeter.

Variation in Volumes of Alkali and Methyl Orange Solutions and in Solvent Composition—While alteration in the volume of methyl orange solution had only a small effect, increasing the volume of alkali decreased the response considerably (Table III). This is probably due to the solubility of the drug base in the dilute aqueous alkali.

In general, the greater the proportion of ethylene chloride in the organic

phase, the greater the spread between blank and drug values. However, since the blank becomes undesirably large if the solvent contains much more than 50 per cent ethylene chloride, it is doubtful that any real advantage would result from the routine use of other than the 1:1 mixture.

Temperature—Experiments performed at 10° and at 24° gave results within 15 per cent of one another, which is the range obtained in different experiments at 24°. It appeared, however, that at 10° the response was

TABLE IV

Response to Different Drugs

1 γ of each drug in organic phase; 16 hours rocking.

Drug	Mol. wt.	Optical density	Optical density per micromole
Cinchonine.....	294.4	0.322	95
Diparcol HCl*.....	334.9	0.071	24
Methadone HCl.....	345.9	0.213	74
Neoantergan maleate.....	401.5	0.177	71

* 10-(2-Dimethylaminoethyl)phenothiazine hydrochloride

TABLE V

Recovery of Drugs from Dog Plasma

0.5 γ of drug extracted; 80 per cent of extract in cell.

Drug	Optical density		
	From H ₂ O	From 1 ml. plasma	From 4 ml. plasma
Neoantergan maleate.....	0.077	0.074	0.062
Methadone HCl.....	0.103	0.102	0.104
Cinchonine.....	0.146	0.139	0.146
Diparcol HCl.....	0.026	0.020	0.022

somewhat greater than at 24°, and that the results were more consistent from cell to cell. At 36°, the results became quite erratic. Because of these experiences, and because the effects of changes in temperature during a determination are not known, the apparatus has been routinely kept in a room maintained at 24° \pm 2°.

Nature of Drug—The response of the method (16 hours shaking) to four drugs is shown in Table IV. In comparison with methadone and neoantergan, cinchonine was more efficient, diparcol less efficient in the transport of methyl orange. The method appears to have general application, but its sensitivity will depend upon properties of the drug.

Treatment of Cells—It is difficult to obtain reliable results with less than 0.1 γ of drug. Chance contamination is difficult to avoid, particularly during the extraction of drugs from plasma. Adsorption of drugs on the glass shaking cells may be a complicating factor. It was found that the most consistent results were obtained if, after being thoroughly cleaned and dried, the cells were used once with standard solutions of the drug under test, and thenceforth washed with warm water only.

Extractability of Drugs—The data in Table V show that the four drugs which we have studied were extracted from plasma about as efficiently as from water by the ethylene chloride-carbon tetrachloride mixture. Plasma blanks were no greater than water blanks. If it is known that recoveries of a drug from water and from plasma are equal, water standards may be run with each set of determinations in place of the plasma standards specified above.

DISCUSSION

The dye method for the determination of basic drugs lacks specificity but has attained wide popularity because of its simplicity and general applicability. The micromodification will be of value for the estimation of low plasma concentrations of drugs and for the determination of higher concentrations when small amounts of blood are available.

The direct method, *i.e.*, utilization of plasma-methyl orange solutions in the continuous extraction vessels, with elimination of the preliminary extraction step, is not applicable in the micromethod if comparatively large volumes of plasma are used. Plasma proteins bind the dye, and troublesome emulsions form. However, for the assay of solutions low in or devoid of proteins, application of the direct method is possible.

The solution of drug in organic solvent is exposed to extraction by aqueous phases in the continuous extraction vessels. It is clear that the partition coefficient of the drug between aqueous and organic phases is more critical in the microprocedure than in the macroprocedure, in which multiple extraction can be used to overcome unfavorable partition.

SUMMARY

A modification of the dye method for the determination of basic drugs in plasma is described. Increased sensitivity is obtained by the continuous transference of methyl orange from a buffer to dilute alkali by means of a solution of the drug in an organic solvent. Depending upon the time allowed for transference, 10 to 50 moles of methyl orange may be transported per mole of drug.

The method has been applied to the determination of neoantergan in blood plasma of dogs. Plasma concentrations reached values of 0.4, 0.9, and 1.8 γ per ml. 1 to 2 hours after oral doses of 2, 5, and 10 mg. per kilo.

BIBLIOGRAPHY

1. Lehman, R. A., and Aitken, T., *J. Lab. and Clin. Med.*, **28**, 787 (1942).
2. Scott, C. C., and Chen, K. K., *J. Pharmacol. and Exp. Therap.*, **87**, 63 (1946).
3. Cronheim, G., and Ware, P. A., *J. Pharmacol. and Exp. Therap.*, **92**, 98 (1948).
4. Brodie, B. B., and Udenfriend, S., *J. Biol. Chem.*, **158**, 705 (1945).
5. Brodie, B. B., Udenfriend, S., and Dill, W., *J. Biol. Chem.*, **168**, 335 (1947).
6. Eggerth, A. H., Littwin, R. J., and Deutsch, J. V., *J. Bact.*, **37**, 187 (1939).

THE UTILIZATION OF D-METHIONINE BY LACTOBACILLUS ARABINOSUS 17-5*

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The microbiological determination of methionine with *Lactobacillus arabinosus* 17-5 has been investigated in several laboratories (2-5). Horn *et al.* (5) employed L-methionine for the standard in their assays, but a DL-methionine standard has been employed by most other investigators, since the synthetic compound is more readily obtained in a high state of purity. It has been reported that DL-methionine is approximately one-half as active as L-methionine and that D-methionine has negligible activity for *L. arabinosus* (3, 4, 6). It is of interest that D-methionine is inactive for *Leuconostoc mesenteroides* P-60 (3, 7) and *Streptococcus faecalis* R (7, 8), but is equally as active as L-methionine for *Lactobacillus fermenti* 36 (3). More recent work (unpublished) from the authors' laboratory has shown that the response of *L. arabinosus* to L-methionine, DL-methionine, and D-methionine is approximately equal in enriched synthetic media. The present report is concerned with the nutritional factors essential for the utilization of D-methionine by *L. arabinosus* 17-5. A comparable study of the utilization of D-glutamic acid by *L. arabinosus* has been reported previously (9).

EXPERIMENTAL

The amino acid composition of the basal medium was the same as that of Shankman's Medium b (10), except that methionine was omitted and the cystine was replaced with 5 mg. per cent (final concentration) of L-cysteine hydrochloride.¹ The composition of the non-amino acid portion of the basal medium was essentially the same as that employed previously for the determination of glutamic acid, isoleucine, leucine, and valine with *L. arabinosus* (11). The total composition of the basal medium is given in Table I. Pyridoxine, pyridoxamine, pyridoxal, folic acid, choline, inositol, and xanthine were omitted from the basal medium individually or in

* Paper 64. For Paper 63, see Camien and Dunn (1). This work was aided by grants from the American Cancer Society through the Committee on Growth of the National Research Council. The authors are indebted to Samuel Eiduson and Gene Molene for technical assistance.

¹ This relatively low concentration was employed, since methionine is readily synthesized by *L. arabinosus* at higher cystine or cysteine levels in this medium (3).

groups in different experiments. In other experiments glycine, serine, and proline (10 mg. per cent of each) were added to the basal medium. The inoculum medium was the same as the basal medium (Table I), except that the amino acids were replaced by vitamin-free casein hydrolysate 750 mg. per cent, L-cysteine hydrochloride 20 mg. per cent, and L-tryptophan 5 mg. per cent. Nutrients omitted from the basal medium in different experiments were likewise omitted from the inoculum medium to

TABLE I

*Basal Medium for Determining Response of L. arabinosus 17-5 to L-, DL-, and D-Methionine**

Constituent		Constituent		Constituent	
	mg. per cent		per cent		γ per cent
DL-Alanine	20	Glucose	2	Thiamine·HCl	100
L-Arginine·HCl	5	Sodium acetate	1.2	Pyridoxine†	160
L-Asparagine	40	Ammonium chloride	0.6	Pyridoxamine·- 2HCl†	10
L-Cysteine·HCl	5			Pyridoxal·HCl†	10
			mg. per cent		
L-Glutamic acid	40	Adenine sulfate	1.2	Ca dl-panto- thenate	200
L-Histidine·HCl·- H ₂ O	5	Guanine	1.2	Riboflavin	200
DL-Isoleucine	20	Uracil	1.2	Niacin	200
L-Leucine	20	Xanthine†	1.2	Biotin	0.5
L-Lysine·HCl	20	KH ₂ PO ₄	50	p-Aminobenzoic acid	10
DL-Phenylalanine	10	K ₂ HPO ₄	50	Folic acid†	0.5
DL-Threonine	20	MgSO ₄ ·7H ₂ O	20	Choline chloride†	1000
L-Tryptophan	3.3	FeSO ₄ ·7H ₂ O	1	Inositol†	2500
L-Tyrosine	3.3	MnSO ₄ ·H ₂ O	1		
DL-Valine	20	NaCl	52		

* The final concentrations of the constituents are given.

† Omitted in some experiments.

avoid a carry-over of these nutrients with the inoculum. The centrifuged and once washed cells from an 18 hour culture were resuspended in and diluted until barely turbid with sterile physiological saline solution. Approximately 0.05 ml. of the suspension was used to inoculate each test-tube (13 × 100 mm.). L-Methionine, DL-methionine, and D-methionine²

² The L-methionine ($[\alpha]_D^{24.59} = +23.18^\circ$ in 1.7 N HCl) and DL-methionine were analytically pure products of Amino Acid Manufactures, University of California, Los Angeles. The D-methionine ($[\alpha]_D^{25} = -23.5^\circ$ in 1 N HCl) was obtained from the H. M. Chemical Company, Ltd., Los Angeles.

were tested in triplicate tubes (3 ml. of final volume per tube) at final concentrations from 0.33 to 4.67 γ per ml. The experimental results are given in Tables II and III and Figs. 1 and 2.

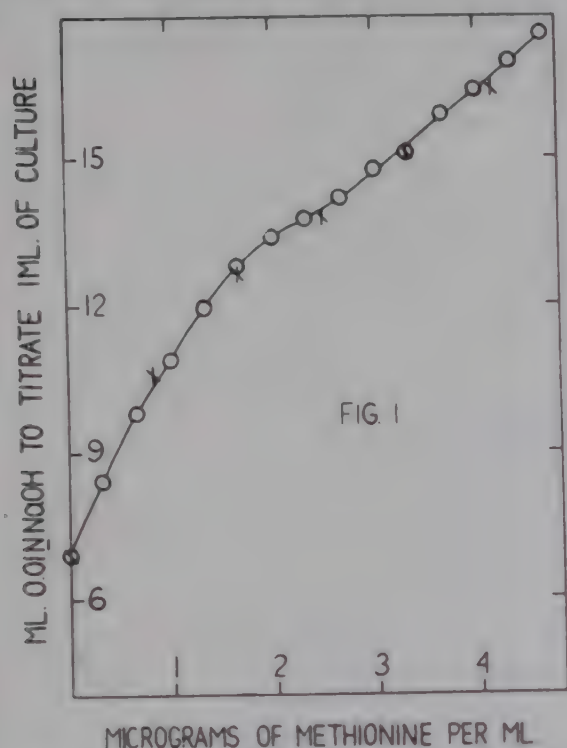


FIG. 1. Response of *L. arabinosus* 17-5 to L-methionine (O) and DL-methionine (X) in the basal medium given in Table I. Each point represents the average titration from triplicate tubes.

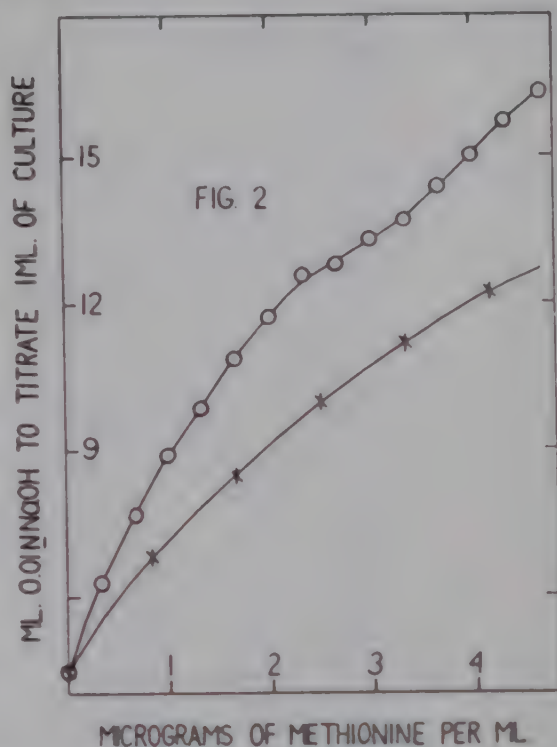


FIG. 2. Response of *L. arabinosus* 17-5 to L-methionine (O) and DL-methionine (X) in the basal medium (Table I) modified to contain no vitamin B₆. Each point represents the average titration from triplicate tubes.

DISCUSSION

It may be seen (Fig. 1) that the response of *L. arabinosus* 17-5 to DL-methionine was nearly identical with the response to L-methionine in the basal medium given in Table I. This result was in disagreement with the reports from other laboratories (4, 6) as well as with earlier work from this laboratory (3). Pyridoxal, pyridoxamine, folic acid, choline, inositol, and xanthine were individually omitted from the basal medium in preliminary experiments, since these substances were lacking in the medium used previously for the determination of methionine with *L. arabinosus* (3). It may be seen (Fig. 2) that the response of this bacterium to DL-methionine was approximately half the response to L-methionine in the basal medium (Table I) modified to contain no vitamin B₆. The relative response to L- and DL-methionine was unchanged, however, by omitting folic acid, choline, inositol, and xanthine from the basal medium. Additional tests (Table II) showed that the presence of either pyridoxamine or pyridoxal in the basal medium permitted D-methionine to be utilized as readily as

TABLE II
Response of *L. arabinosus* 17-5 to L-Methionine and D-Methionine at Different Concentrations of Pyridoxine, Pyridoxal, and Pyridoxamine

Methio- nine added <i>γ</i> per ml.	Form and amount of vitamin B ₆ added, <i>γ</i> per cent															
	None		160 (A)*		16 (A)*		10 (B)*		1 (B)*		10 (C)*		1 (C)*		†	
	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D
Form of methionine added																
0	2.68		3.32		3.10		5.37		4.89		5.20		4.49		5.08	
0.83	6.50	2.82	6.42	3.33	6.54		8.99	8.96	8.68	8.79	8.70	8.79	7.68	5.99	8.65	8.72
1.67	8.81	2.92	8.68	3.41	8.90		11.13	11.20	10.62	11.00	10.81	10.92	9.87	6.52	10.79	11.03
2.50	10.44	3.18	10.16	3.57	10.74		12.69	12.69	12.40	12.43	12.39	12.57	11.73	6.71	12.40	12.51
3.33	11.67	3.39	11.18	3.68	11.63		13.61	13.56	13.60	13.39	13.50	13.44	12.80	6.90	13.33	13.31
4.17	12.07	2.91	11.51	3.81	12.39		15.27	15.09	15.07	14.92	13.71	13.89	13.33	6.98	14.89	14.89

* Ml. of 0.01 N NaOH to titrate 1 ml. of culture. Each value is the average of triplicate determinations. The medium was that given in Table I, except that pyridoxine, pyridoxamine, and pyridoxal were omitted. Pyridoxine (A), pyridoxamine (B), and pyridoxal (C) were added aseptically to the previously sterilized assay tubes.
† Results with the unaltered basal medium (Table I).

L-methionine by *L. arabinosus*. Pyridoxamine appeared to be about 10 times as active as pyridoxal in promoting the utilization of D-methionine, whereas pyridoxine was entirely inactive in this respect at 160 times the minimum effective dose of pyridoxamine. The results given in Table III indicated that folic acid, choline, inositol, xanthine, glycine, serine,

TABLE III

*Response of L. arabinosus 17-5 to L-Methionine and D-Methionine at Different Concentrations of Pyridoxamine and in Two Different Media**

Methionine added	Final concentration of pyridoxamine·2HCl, γ per cent							
	0		0.04		0.2		1	
	Form of methionine added							
γ per ml.	L	D	L	D	L	D	L	D
Medium I								
0	2.60		3.41		4.53		6.22	
0.83	6.82	2.69	7.59	4.29	8.58	7.70	9.79	9.98
1.67	9.58	2.89	10.39	4.22	11.04	8.67	12.30	12.39
2.50	11.40	3.10	12.52	4.28	13.00	8.98	13.92	14.09
3.33	12.84	3.36	13.70	4.47	14.08	9.34	15.03	14.98
4.17	13.21	3.43	14.74	4.68	15.33	9.39	16.39	16.32
Medium II								
0	2.49		3.29		4.59		6.47	
0.83	6.09	2.38	7.19	3.79	8.20	7.29	9.70	9.79
1.67	7.92	2.68	9.98	3.79	10.66	8.21	12.01	12.21
2.50	8.78	2.88	12.18	3.82	12.52	8.90	13.88	13.76
3.33	8.91	3.08	13.70	3.94	13.91	8.72	14.84	14.83
4.17	8.91	3.21	14.49	4.10	15.08	9.08	16.20	16.14

* Ml. of 0.01 N NaOH to titrate 1 ml. of culture. Each value is the average of three determinations. Medium I was that given in Table I, except that pyridoxine, pyridoxamine, and pyridoxal were omitted and glycine, DL-serine, and L-proline were added (final concentration 10 mg. per cent of each). Medium II was that given in Table I, except that pyridoxine, pyridoxamine, pyridoxal, folic acid, choline, inositol, and xanthine were omitted.

and proline exerted a sparing action on pyridoxamine in promoting the utilization of L-methionine by *L. arabinosus*. This sparing effect appeared to be negligible in promoting the utilization of D-methionine, however (Table III). The lack of significant stimulation by the added nutrients in the presence of an adequate concentration of pyridoxamine (Table III) suggested that the complete basal medium may be able to resist stimulatory effects of similar substances in assay samples.

Riesen *et al.* (4) have reported that D-methionine was essentially inactive for *L. arabinosus* in a basal medium containing oxidized casein hydrolysate and a relatively complete array of additional nutrients, including a high concentration of pyridoxamine. It seems likely, therefore, that oxidized casein hydrolysate may contain substances capable of preventing the utilization of D-methionine by *L. arabinosus* even in the presence of relatively large amounts of pyridoxamine. That the oxidation products of methionine may interfere with the utilization of D-methionine might be expected, since the utilization of D-glutamic acid by *L. arabinosus* is completely suppressed by relatively high concentrations of aspartic acid, the 4-carbon homologue of glutamic acid (9).

L. arabinosus appears to utilize D-methionine by converting it quantitatively to L-methionine in the presence of pyridoxamine or pyridoxal. It does not seem unlikely that this conversion is catalyzed by an enzyme system which requires pyridoxamine or one of its derivatives as a coenzyme. A similar function of pyridoxal in the racemization of L-alanine by *L. casei* and other lactic acid bacteria has been postulated by Holden and Snell (12). It is not entirely clear, however, that the required D-alanine is produced from L-alanine, since neither form of alanine is required by these organisms in the presence of an adequate concentration of vitamin B₆ (13). That pyridoxamine may promote the utilization of the D forms of other amino acids has been reported by Lyman and Kuiken (14), who found that D-isoleucine and D-leucine were partially able to replace the L forms with *L. arabinosus* when pyridoxamine was added to the basal medium.

It is of interest that the cells of *L. arabinosus* 17-5 have been reported to contain 3.7 to 4.0 per cent D-glutamic acid (15, 16) and 1.2 to 2.1 per cent D-alanine (12),³ indicating that these "unnatural" enantiomorphs may be essential metabolites for this organism. It does not appear that D-methionine has a similar function in the metabolism of *L. arabinosus*, since significant amounts of D-methionine were not found in the cells of this bacterium (unpublished results).

It has been reported that *L. fermenti* 36 utilized DL-methionine equally as well as L-methionine in a medium containing no added pyridoxamine or pyridoxal (3). Further experiments are needed, however, to rule out the effects of these vitamins, since they may be produced in significant amounts by autoclaving pyridoxine together with the other medium constituents (17). *L. mesenteroides* P-60 utilized only L-methionine (3) in a modification of Medium D, Paper XVIII (18). It has since been found (unpublished data) that the utilization of D-methionine by *L. mesenteroides* P-60 is not promoted by further modifying this medium to contain pyri-

³ The value, 2.0 per cent, was found for D-alanine in dried cells of *L. arabinosus* in the authors' unpublished experiments.

doxamine, pyridoxal, choline chloride, inositol, and xanthine in the concentrations given in Table I of the present paper. It appears, therefore, that *L. mesenteroides* P-60 may be a satisfactory test organism for the determination of L-methionine and that D-methionine may be determined by difference with *L. arabinosus* or *L. fermenti* to determine the total of the methionine enantiomorphs. Studies on the determination of L- and D-methionine in normal and cancerous rat tissues and on the utilization of D-methionine by a variety of lactic acid bacteria are in progress.

SUMMARY

Lactobacillus arabinosus 17-5 has been shown to utilize D-methionine and DL-methionine equally as well as L-methionine in a synthetic medium containing at least 1 γ per cent of pyridoxamine or 10 γ per cent of pyridoxal. At lower concentrations of these vitamins the utilization of D-methionine was reduced or eliminated. Pyridoxine and other nutrients in relatively high concentrations were ineffective in promoting utilization of D-methionine. The significance of these results in relation to previously published work has been discussed.

BIBLIOGRAPHY

1. Camien, M. N., and Dunn, M. S., unpublished data.
2. Shankman, S., Dunn, M. S., and Rubin, L. B., *J. Biol. Chem.*, **150**, 477 (1943).
3. Dunn, M. S., Camien, M. N., Shankman, S., and Block, H., *J. Biol. Chem.*, **163**, 577 (1946).
4. Riesen, W. H., Schweigert, B. S., and Elvehjem, C. A., *J. Biol. Chem.*, **165**, 347 (1946).
5. Horn, M. J., Jones, D. B., and Blum, A. E., *J. Biol. Chem.*, **166**, 321 (1946).
6. Stokes, J. L., and Gunness, M., *J. Biol. Chem.*, **154**, 715 (1944).
7. Lyman, C. M., Moseley, O., Butler, B., Wood, S., and Hale, F., *J. Biol. Chem.*, **166**, 161 (1946).
8. Stokes, J. L., Gunness, J., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, **160**, 35 (1945).
9. Camien, M. N., and Dunn, M. S., *J. Biol. Chem.*, **179**, 935 (1949).
10. Shankman, S., *J. Biol. Chem.*, **150**, 305 (1943).
11. Dunn, M. S., Camien, M. N., Malin, R. B., Murphy, E. A., and Reiner, P. J., *Univ. California Pub. Physiol.*, **8**, 293 (1949).
12. Holden, J. T., and Snell, E. E., *J. Biol. Chem.*, **178**, 799 (1949).
13. Dunn, M. S., Shankman, S., Camien, M. N., and Block, H., *J. Biol. Chem.*, **168**, 1 (1947).
14. Lyman, C. M., and Kuiken, K. A., *Federation Proc.*, **7**, 170 (1948).
15. Dunn, M. S., Camien, M. N., Shankman, S., and Block, H., *J. Biol. Chem.*, **168**, 43 (1947).
16. Camien, M. N., Salle, A. J., and Dunn, M. S., *Arch. Biochem.*, **8**, 67 (1945).
17. Snell, E. E., *Proc. Soc. Exp. Biol. and Med.*, **51**, 356 (1942).
18. Dunn, M. S., Shankman, S., Camien, M. N., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, **156**, 703 (1944).

THE METABOLIC FATE OF ACETYLDEHYDROTYROSINE IN THE RAT*

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In the present study, an effort was made to determine the extent to which acetyldehydrotyrosine labeled with N^{15} would be metabolized by the rat. It was hoped that the result of such an experiment would throw further light on the possible rôle of derivatives of α, β -unsaturated amino acids in protein metabolism (1-3). Isotopic acetyldehydrotyrosine was chosen because it can readily be synthesized by the method of Bergmann and Stern (2) from isotopic glycine, and also because previous studies from this laboratory had shown this dehydroamino acid derivative to be converted by growing cultures of *Escherichia coli* to a nitrogenous phenol of undetermined nature (3). A *tyrosineless* strain of *E. coli*, however, could not use acetyldehydrotyrosine in place of L-tyrosine for growth (4).

Three Sprague-Dawley male rats (weight, 320 to 330 gm.) were fasted for 24 hours, and then received, at hourly intervals, five intraperitoneal injections of 42.5 mg. of acetyldehydrotyrosine $\cdot H_2O$ (32.29 atom per cent excess N^{15}) dissolved in 3 cc. of water containing 1 equivalent of sodium bicarbonate. Each rat thus received 212.5 mg. of the compound. The urine was collected in the presence of 3 cc. of 2 N sulfuric acid for exactly 24 hours from the time of the first injection, pooled, and filtered to remove insoluble material. This insoluble residue was extracted with N sodium bicarbonate, the extract was filtered, and the volume of the filtrate was adjusted to 200 cc. with water; analysis of the solution showed it to contain 73.6 mg. of nitrogen. The N^{15} concentration of the solution was 1.602 atom per cent excess; this corresponds to 0.60 mg. of N^{15} . The filtered urine was diluted to 500 cc. with water, and aliquots of the solution were used for determinations of total nitrogen, urea nitrogen, and ammonia nitrogen, as well as the corresponding N^{15} concentrations. The results of these analyses are presented in Table I, and show that at least 93 per cent of the administered N^{15} was voided during the collection period. The low isotope concentration of the urinary urea and ammonia indicates that little, if any, of the acetyldehydrotyrosine entered the metabolic pool of amino acids. It would appear, therefore, that, under the condi-

* This study was aided by grants from the James Hudson Brown Fund of the Yale University School of Medicine, and from the Rockefeller Foundation.

tions of this experiment, the α,β -unsaturated amino acid derivative was not utilized by the fasting rat to an appreciable extent for its protein metabolism.

The fact that this resistance to metabolic utilization is due to the presence of the α,β double bond is illustrated by comparison of the above data with the results of a parallel experiment in which isotopic acetyl-DL-tyrosine (32.19 atom per cent excess N^{15}) was administered to fasting rats under the same experimental conditions as those described above. Each rat received 371 mg. of the compound, which was prepared by the catalytic hydrogenation of isotopic acetyldehydrotyrosine in the presence of palladium black. As was to be expected from earlier studies on the metabolism of acetyl amino acids (5), the nitrogen of acetyltyrosine appeared

TABLE I
Administration of Isotopic Acetyldehydrotyrosine and of Acetyl-DL-tyrosine to Fasting Rats

All quantities are given on the basis of groups of three rats.

Compound	N ¹⁵ admin- istered	Urinary constituents						
		Total N			Ammonia N		Urea N	
		Amount	N ¹⁵ con- centration	Total N ¹⁵	Amount	N ¹⁵ con- centration	Amount	N ¹⁵ con- centration
	mg.	mg.	atom per cent excess	mg.	mg.	atom per cent excess	mg.	atom per cent excess
Acetyldehydrotyro- sine · H ₂ O	12.37	605*	1.915*	11.58*	35.4	0.122	332	0.015
Acetyl-DL-tyrosine	23.2	472	2.934	13.85	25.7	0.578	337	0.823

* These figures include the values for the bicarbonate extract of the urinary sedi-
ment and the filtered urine.

in the urinary urea and ammonia to an appreciable extent, and a significant portion of the isotopic nitrogen had been retained in the tissues at the end of the 24 hour collection period. This suggests that the fasting rat is incapable of reducing the α,β double bond of acetyldehydrotyrosine at a rate sufficiently great to permit the metabolic utilization of this substance. From previous work (3), it would appear that *E. coli* also is incapable of performing this hydrogenation. These conclusions as to the metabolic rôle of α,β -unsaturated amino acid derivatives are consonant with recent data (6) on the metabolism of leucine labeled with deuterium in the α , β , and γ positions.

It is of interest that, following the administration of isotopic acetyl-DL-tyrosine, the ratio of the N^{15} concentration in the urinary ammonia to the N^{15} concentration in the urinary urea is 0.70. Although this value

is greater than that recently reported after the administration of isotopic L-aspartic acid to fed rats (7), it is considerably lower than those cited for other amino acids, including DL-tyrosine (for literature citations, see Wu and Rittenberg (7)).

SUMMARY

Following the administration of acetyldehydrotyrosine, labeled with N^{15} , to fasting rats, the isotope concentration of the urinary ammonia and urea is low, and 93 per cent of the administered N^{15} is excreted in the urine within 24 hours. It has been concluded, therefore, that, under the conditions of this experiment, the dehydroamino acid derivative is not utilized to an appreciable extent for the protein metabolism of the fasting rat.

BIBLIOGRAPHY

1. Dakin, H. D., *J. Biol. Chem.*, **67**, 341 (1926).
2. Bergmann, M., and Stern, F., *Ann. Chem.*, **448**, 20 (1926).
3. Fruton, J. S., Simmonds, S., and Smith, V. A., *J. Biol. Chem.*, **169**, 267 (1947).
4. Simmonds, S., Tatum, E. L., and Fruton, J. S., *J. Biol. Chem.*, **169**, 91 (1947).
5. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **169**, 467 (1947).
6. Sprinson, D. B., and Rittenberg, D., *Federation Proc.*, **7**, 191 (1948).
7. Wu, H., and Rittenberg, D., *J. Biol. Chem.*, **179**, 847 (1949).

ISOLATION OF ADRENAL CORTICAL HORMONES FROM URINE: 17-HYDROXYCORTICOSTERONE AND 17-HYDROXY-11- DEHYDROCORTICOSTERONE

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It has been amply demonstrated (1-7) that there is present in normal urine material with glycogenic activity similar to that of some of the adrenal cortical hormones which have an oxygen atom at C-11. The exact nature of this active material has not been determined. Although some unknown adrenal hormone or a metabolite of some known hormone may be responsible for this activity, it has seemed more likely that one (or more) of the known adrenal cortical hormones is excreted in the urine and is responsible for the observed activity. Several chemical methods (8-11) for the determination of "corticosteroids" have been based on the likelihood that any compound with the physiologic properties of an adrenal cortical hormone would have the characteristic α -ketol side chain. Although not entirely specific, the reaction of urinary extracts with periodic acid to give formaldehyde, a property of a primary α -ketol group, has indicated the presence of such "formaldehydogenic" compounds (10, 11).

It was found recently that when large amounts of formaldehydogenic material were excreted in a case of Cushing's syndrome 17-hydroxycorticosterone could be isolated from the urine (12) (Fig. 1). This successful isolation of an adrenal cortical hormone from the urine stimulated an attempt to isolate similar compounds from postoperative urine, which has been shown to contain an increased amount of biologically active material. Further studies were carried out on urine obtained from patients suffering from rheumatoid arthritis, who were treated with adrenocorticotrophic hormone, and from patients treated with relatively large amounts of 17-hydroxy-11-dehydrocorticosterone and its acetate (13). It is the purpose of this paper to describe the isolation of adrenal cortical hormones from the urine of these patients and from postoperative urine.

Postoperative Urine

In this study¹ urine was obtained from patients during the 5 days immediately following operation. The specimens were pooled in the refrigerator with toluene as a preservative and eventually were concentrated

¹ I am indebted to Dr. R. G. Sprague who made the arrangements for collection of urine used in this study and in the study of patients with Addison's disease.

in a vacuum and extracted with chloroform. The urine residue was adjusted to pH 1 and, after standing for 24 hours, it was again extracted with chloroform. These extracts were kept separate at first with the idea of determining whether the acidification had liberated some compound or compounds different from those present in the free state. Actually, the amount of formaldehydogenic substances in the extract obtained after addition of acid was so small in comparison with that obtained before addition of acid that no attempt was made to isolate anything from this small fraction. Later it was combined with one of the major fractions. The toluene used as a preservative extracted very little formaldehydogenic material.

After approximately 100 liters of urine had been extracted, the extract was carried through a partial fractionation. In this first attempt at

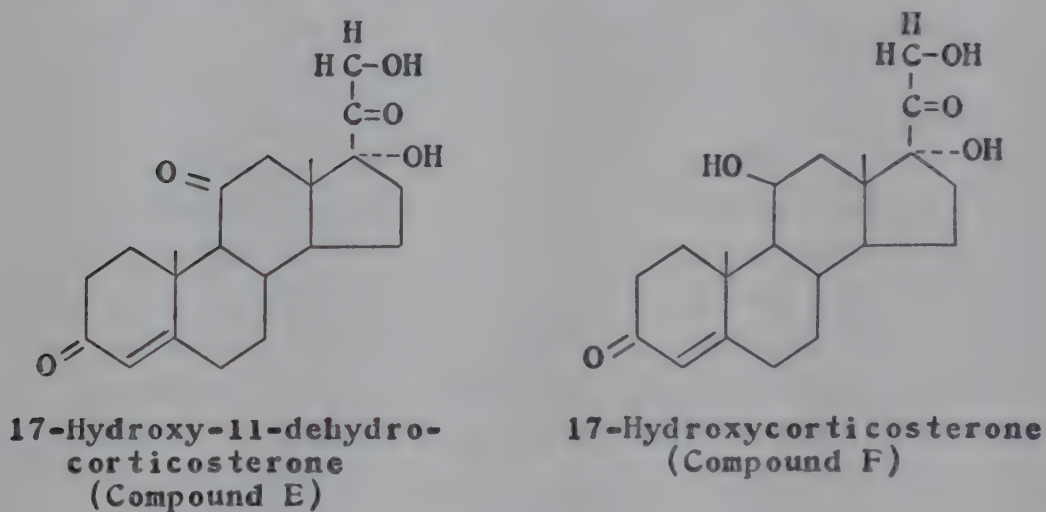


FIG. 1

isolation the losses were evidently rather large. With the information thus obtained as a guide, the extract of the second part of the pool obtained from unacidified urine, which contained approximately 110 mg. of formaldehydogenic material, was subjected to repeated distribution between benzene and water as described in the experimental part of this paper. The material thus obtained finally in aqueous solution was extracted with chloroform, combined with a similar fraction from the extract of the total urine after acidification to pH 1, and was treated in methanol solution with Girard's Reagent T (14) and acetic acid. The Girard complex was decomposed fractionally as described by Reichstein (15).

The ketonic fractions so obtained were diluted with 95 per cent alcohol to concentrations of 4.7 to 6.0 mg. per 100 ml. and their absorption in the region 234 to 244 $m\mu$ was examined with a Beckman spectrophotometer. The ketonic fraction which was liberated when the solution of the Girard complex was acidified with hydrochloric acid until it turned Congo

red paper a pure blue was the only fraction which showed an absorption maximum in this region. From the extinction at 239 $m\mu$, assuming a molecular weight of 360 and a molar extinction coefficient of 16,000 for the material absorbing maximally at this wave-length, it was calculated that 28 per cent of this fraction, or 37 mg., was an α,β -unsaturated ketone, probably of the nature of 17-hydroxy-11-dehydrocorticosterone. This fraction was combined with two similar fractions obtained from the first half of the urine pool which weighed 26 mg. and apparently contained 15 mg. of α,β -unsaturated ketone. Attempts to obtain crystals from these combined fractions by treatment with small amounts of chloroform were unsuccessful. Therefore, the solvent was removed and the residue was acetylated with acetic anhydride and pyridine. Crystals were obtained from methanol which gave a green fluorescence with concentrated sulfuric acid, a reaction which is given by 17-hydroxycorticosterone and its acetate. However, after recrystallization the acetate melted at 233-234° and no longer gave the fluorescence with sulfuric acid. It reduced ammoniacal silver promptly in the cold but did not form an insoluble dinitrophenylhydrazone. These properties indicate the presence of an α -ketol side chain and absence of a 3-keto group.

The mixture of the acetates, which remained in the mother liquors, was subjected to chromatographic analysis on a column of magnesium silicate with mixtures of benzene and petroleum ether, benzene and ethyl ether, and benzene and alcohol. The fraction eluted with benzene-ether, 4:1, proved to be identical with the material which was obtained by direct crystallization from methanol. The next crystalline fraction, which was eluted with benzene-ether, 1:1, melted at 245-250° and gave a green fluorescence with concentrated sulfuric acid. The melting point was depressed about 20° by 17-hydroxy-11-dehydrocorticosterone acetate (m.p. 237-239°). A fraction which weighed 4 mg. was eluted with dry ether. After recrystallization from methanol it melted at 220-222°, a mixture with 17-hydroxycorticosterone acetate (m.p. 218-220°) melted at 218-220°, and it gave a strong green fluorescence with sulfuric acid. These properties indicated that this fraction was 17-hydroxycorticosterone acetate, but the small amount of material precluded a more certain identification.

A fourth fraction was eluted with benzene containing 5 per cent of alcohol. After recrystallization from ethanol and methanol it melted at 225-228° and also gave a green fluorescence with concentrated sulfuric acid. This fraction reduced ammoniacal silver promptly in the cold and gave a red dinitrophenylhydrazone. These properties indicated the presence of an α -ketol side chain and an α,β -unsaturated ketone group as well as an 11-hydroxyl group. Assuming that the structural features indicated are in the positions usual in the adrenal steroids, the minimal

structural requirements for these properties would be those of corticosterone, but the acetate of this hormone melts at 152–153°. Reichstein's Substance E (Δ^4 -pregnene-11 β ,17 α ,20,21-tetrol-3-one, m.p. 229–230°) was considered as a possibility, but it does not reduce alkaline silver in the cold (16). The urinary substance possibly is more highly oxygenated than 17-hydroxycorticosterone, since it was removed from the column only when a fairly high concentration of alcohol in benzene was used, whereas the fraction with the properties of 17-hydroxycorticosterone was eluted with ether.

Thus, the fraction tentatively identified as 17-hydroxycorticosterone acetate was the only fraction with the properties of the acetate of any of the known adrenal cortical steroids.

Patients Treated with Adrenocorticotrophic Hormone

The urine of three women (Cases 1, 2, and 3) and one man (Case 4) with rheumatoid arthritis who had received adrenocorticotrophic hormone (ACTH) was made available.² The ACTH³ was given in doses of 100 mg. per day for 12 days. During this period there was an increase in the formaldehydogenic substances which, toward the end of the period of administration, reached peaks of approximately 12, 17, and 7 mg. in the case of the women, and 6 mg. per day in the case of the man. Since part of the urine was used for other purposes, extracts were available which in Cases 1 and 2 contained approximately 50 mg. of formaldehydogenic substances. In Case 1 the pool of extracts in 10 per cent alcohol was concentrated under reduced pressure, with a bath temperature less than 50°, in order to remove the alcohol, and the material was distributed between benzene and water five times according to the general procedure described in the experimental part. The benzene was removed from the final fraction and the residue was crystallized from a little chloroform. The crystals weighed 9.5 mg., melted at 208–210°, gave a strong green fluorescence with concentrated sulfuric acid, and immediately reduced ammoniacal silver. After recrystallization from methanol the material melted at 214–216° and the melting point of a mixture with 17-hydroxycorticosterone was not depressed. Approximately half of the crystals was acetylated with acetic anhydride and pyridine at room temperature. After crystallization from methanol the melting point of the acetate was 214–216°, and it was not depressed by admixture of 17-hydroxycorticosterone acetate.

² I am indebted to Dr. P. S. Hench, Dr. C. H. Slocumb, and Dr. H. F. Polley for their cooperation in making available this urine and that of other patients treated with 17-hydroxy-11-dehydrocorticosterone.

³ The ACTH was made available through the courtesy of Dr. John R. Mote, the Armour Laboratories, Chicago, Illinois.

Fractionation of the material in the chloroform mother liquor with the aid of Girard's Reagent T and treatment of the ketonic fraction which was liberated upon addition of sufficient hydrochloric acid to turn Congo red paper blue with acetone and petroleum ether gave a few more crystals which had the same properties as those obtained by direct crystallization from chloroform. Acetylation of the non-crystalline material in the mother liquor and crystallization from methanol-water yielded a few crystals of the acetate. The amount of additional crystalline material obtained by these procedures was estimated to be not more than 1 mg.

It was thought that the rather low yield of crystalline material in the first case may have resulted from losses during the distribution between benzene and water and during the later treatment. Consequently the pool of extracts in Case 2, after removal of alcohol, was subjected to only two distributions between water and benzene. The final aqueous solution was extracted with chloroform, and the extract was filtered and concentrated at a low temperature in a vacuum to a small volume. Crystals began to separate when the glass was scratched with a spatula. After refrigeration overnight the crystals which were washed with chloroform weighed 24.3 mg. After recrystallization from methanol they melted at 212–215° and a mixture with 17-hydroxycorticosterone (m.p. 214–216°) melted at 212–215°. They gave an intense green fluorescence with concentrated sulfuric acid. $[\alpha]_D^{27} = +168^\circ \pm 5^\circ$ ($c = 0.310$ per cent). The acetate melted at 217–219° and the melting point was not depressed by admixture with 17-hydroxycorticosterone acetate which melted at the same point. Since all of these properties agreed with the properties of 17-hydroxycorticosterone, the identification was considered to be complete. No more crystals could be obtained from the chloroform mother liquor. After removal of chloroform the residue was acetylated, but failed to give crystals of any acetate.

In Cases 3 and 4 the procedure was changed somewhat, as detailed in the experimental section. Chloroform solutions of the extracts were submitted to fractionation on a column of a 1:1 mixture of magnesium silicate and Celite. In Case 3, 6.5 mg. were separated from chloroform before the solution was placed on the chromatographic column. Chloroform alone eluted from the column material similar to the non-ketonic fraction obtained previously in the Girard fractionation. It melted at 233°, contained nitrogen, was quite soluble in water, and was identified as caffeine. A 3:1 mixture of chloroform and acetone eluted a fraction from which 17-hydroxycorticosterone could be obtained by crystallization from chloroform. In Case 3, 6 mg. were obtained from this fraction which appeared to contain approximately 15 mg. of 17-hydroxycorticosterone as indicated by measurements of optical activity and absorption at 238 m μ . The total

of 12.5 mg. of 17-hydroxycorticosterone isolated in this case was derived from 37 mg. of formaldehydogenic substances in the original pool of extracts.

In Case 4, 7.5 mg. of 17-hydroxycorticosterone were obtained from 26 mg. of formaldehydogenic substances as determined by assay of the pool of extracts.

Patients Receiving 17-Hydroxy-11-dehydrocorticosterone and Its Acetate

The urine of two patients with Addison's disease and four patients with rheumatoid arthritis who were receiving 17-hydroxy-11-dehydrocorticosterone or its acetate was studied in an effort to identify metabolites of this hormone. Only the isolation of the unchanged hormone will be considered here.

One of the patients with Addison's disease received 100 mg. per day of 17-hydroxy-11-dehydrocorticosterone acetate for 16 days, and the other received 50 mg. of the acetate for 23 days. Extracts of the urine were made daily during the period of administration of the hormone, and for 27 days after administration of the hormone had been stopped in the first case, since quantitative determinations indicated a continued relatively high excretion of formaldehydogenic substances during this time. All of the extracts were combined and the solvent was removed. The residue was distributed once between benzene and water, and the water-soluble fraction of 687 mg. was recovered by extraction with chloroform. It was treated with Girard's Reagent T and the Girard complex of the ketonic fraction was subjected to fractional decomposition. In this case also the major ketonic fraction of 56 mg. was liberated when the acidity of the solution was adjusted to turn Congo red paper blue. This fraction deposited crystals when the chloroform solution was concentrated to a small volume. Two crops of 3.5 mg. each were obtained. The first crop melted at 216–218°. A mixture with 17-hydroxy-11-dehydrocorticosterone (natural, m.p. 218–220°) melted at 216–218°. The crystals reduced ammoniacal silver immediately in the cold and gave a green fluorescence with concentrated sulfuric acid.

The second crop of crystals, which melted at 209–212°, was converted to the acetate with pyridine and acetic anhydride at room temperature. After two crystallizations from methanol it melted at 238–240° and still gave a green fluorescence with concentrated sulfuric acid. A mixture with 17-hydroxy-11-dehydrocorticosterone acetate (m.p. 240–242°) melted at 238–240°.

The urine of four patients, three men and one woman, with rheumatoid arthritis was collected and extracted daily while 100 mg. per day of 17-hydroxy-11-dehydrocorticosterone (Fig. 1) were being administered by

intramuscular injection of an aqueous suspension containing 25 mg. per ml. The urine was collected over a period during which 19.8 gm. of the hormone were administered. The extracts were pooled in the refrigerator and worked up in two portions. Each portion was distributed several times between benzene and water as described in the experimental part. The aqueous fractions were tested for the presence of an α -ketol group by its power to reduce ammoniacal silver in the cold, and for the α,β -unsaturated ketone group by its ability to form a red or orange dinitrophenylhydrazone. These fractions were also assayed for formaldehydogenic substances. The combined aqueous fractions which gave a strong reduction of ammoniacal silver and a red or orange dinitrophenylhydrazone, assayed 124.6 mg. of formaldehydogenic material. The aqueous solution was concentrated and extracted with benzene. The benzene extract was treated with Girard's Reagent T and the ketonic fraction was decomposed fractionally. The ketonic fraction liberated when the solution turned Congo red paper blue weighed 90 mg. and deposited crystals as its chloroform solution was concentrated to a small volume. The crystals weighed 15 mg. and melted at 217–219°. The filtrate, after removal of chloroform and treatment with acetone and petroleum ether, gave a second crop of 6 mg. which melted at 225–226° after recrystallization from methanol. Both crops gave a strong green fluorescence with concentrated sulfuric acid. When the two crops of crystals were combined and recrystallized from methanol, large prisms were obtained which melted at 227–229°. The fluorescent reaction with sulfuric acid was still strong. This material was identified as 17-hydroxy-11-dehydrocorticosterone by its specific rotation ($[\alpha]_D^{25} = +204^\circ \pm 4^\circ$), by observation of an absorption maximum at 240 $m\mu$ ($\epsilon = 16,200$), and by the melting point of its acetate (240–242°) which was not depressed by admixture of 17-hydroxy-11-dehydrocorticosterone acetate (m.p. 240–242°).

The melting point of highly purified synthetic 17-hydroxy-11-dehydrocorticosterone has been found⁴ to be 230–231°, or more than 10° higher than the best melting point reported for the natural compound. As in the case of the compound obtained from adrenal extracts, the compound obtained from urine could not be freed by recrystallization or acetylation from the substance giving the green fluorescence with concentrated sulfuric acid.

Comment

The fraction with the properties of 17-hydroxycorticosterone acetate isolated from postoperative urine was disappointingly small. In working with urinary fractions known to contain this hormone in the free state,

⁴ Private communication from Dr. V. R. Mattox.

one gains the impression that it is very readily destroyed. It is not likely that it separated quantitatively from chloroform in the presence of much other material; yet exhaustive efforts to obtain more from the mother liquor yielded very little. In the case of the postoperative urine it may be that the preliminary concentration of the urine had an adverse effect.

The failure to find any fraction which could be identified as 17-hydroxy-11-dehydrocorticosterone indicates that this substance was not present in significant amounts, since it is more easily isolated than 17-hydroxycorticosterone.

The isolation of 17-hydroxycorticosterone after stimulation of the adrenal cortex with ACTH suggests that this hormone may well be the principal, if not the only, glycogenic steroid which is excreted in the urine normally and under conditions of stress. This suggestion received some support from the isolation of the fraction tentatively identified as 17-hydroxycorticosterone from postoperative urine, although the amount isolated was too small to be very significant.

In the case of 17-hydroxy-11-dehydrocorticosterone isolated from the urine of patients with Addison's disease, the green fluorescence which developed when these crystals were treated with concentrated sulfuric acid indicated the presence of an impurity. This reaction is not specific, but it is given only by compounds with an 11-hydroxyl group. Although early reports (15, 17) on preparations of 17-hydroxy-11-dehydrocorticosterone isolated from adrenal extracts indicated that the green fluorescence developed by concentrated sulfuric acid was a property of this substance, Reichstein and Shoppee (16) pointed out that the completely pure monoacetate does not give the reaction, and it is now evident that the synthetic compound does not give it. Manifestation of the reaction by the crystals isolated from the urine is suggestive of the presence of a small amount of 17-hydroxycorticosterone, which gives the reaction very strongly. It is likely that the presence of this compound was responsible for the reports that 17-hydroxy-11-dehydrocorticosterone isolated from adrenal glands gave this reaction. Several other adrenal steroids also give the reaction, and it is possible that one of these may have been responsible in the case of the material isolated from urine. In any event the indications are that the 11-carbonyl group of some of the 17-hydroxy-11-dehydrocorticosterone administered had undergone reduction to an 11-hydroxyl group. Endogenous production of cortical steroids is improbable in view of the severe Addison's disease of long standing which characterized the patients in this study.

In the case of the patients with rheumatoid arthritis who were treated with 17-hydroxy-11-dehydrocorticosterone, the product isolated also gave a green fluorescence with concentrated sulfuric acid, even though the

melting point approached that of the synthetic hormone. The impurity giving the fluorescent reaction in this instance may have come from the intact adrenals of these arthritic patients, although there was reason to think that their adrenal cortical function was largely inhibited by the administration of 17-hydroxy-11-dehydrocorticosterone.

The study of postoperative urine throws some light on the nature of the glycogenic activity of the urine under conditions of stress, but the small amount of the compound isolated and tentatively identified as 17-hydroxycorticosterone leaves much to be desired. However, when consideration is given also to the isolation of 17-hydroxycorticosterone in satisfactory quantities from the urine of the four patients who received ACTH and the one patient (previously described (12)) with cortical hyperplasia, the importance of this particular hormone in relation to human adrenal function and to the urinary glycogenic activity becomes evident.

EXPERIMENTAL

Collection and Extraction of Urine—The postoperative urine was collected from eight to ten patients at a time and was brought to the laboratory daily, where it was pooled in 5 gallon bottles containing toluene and stored in a cold room. When a bottle was filled, the urine was concentrated to a volume of between 2 and 3 liters by distillation in a vacuum at a bath temperature of not more than 50°. It was necessary to keep the bath temperature less than 50° until the toluene had been removed in order to avoid overheating the urine. After that the urine distilled smoothly while remaining at a relatively low temperature. The concentrate was extracted three times with 500 ml. of chloroform. The emulsions that formed were broken by filtration through a pad of infusorial earth. The extract was washed once with a dilute solution of sodium carbonate and then with water until neutral. The extract of each batch was assayed for formaldehydogenic substances which amounted to approximately 0.7 to 1.5 mg. per liter of urine. The urine residues were adjusted to approximately pH 1 with hydrochloric acid and allowed to stand overnight at room temperature. They were then extracted three times with 500 ml. of chloroform. The amount of formaldehydogenic substances varied considerably but averaged approximately 0.3 mg. per liter of urine.

In all other cases the urine was received at the laboratory daily and the acidity was adjusted to pH 1. It was allowed to stand overnight at room temperature, and then extracted four times with 0.15 volume of chloroform. The extract was washed as described, and part was used for the determination of formaldehydogenic substances. The remainder was stored in the refrigerator.

Concentration and Distillation—In all cases distillations were conducted

in the vacuum produced by an efficient water aspirator. The bath temperature was not allowed to rise above 50°, and when a solution was taken to dryness the temperature was kept under 40°. It is not known with certainty that these precautions are necessary. However, it appears that 17-hydroxycorticosterone may be easily lost. This hormone can be recrystallized readily with the use of heat when it is relatively pure, but in the presence of other urinary constituents heating a chloroform solution makes it very difficult or impossible to obtain crystals, even though the hormone is known to be present. Therefore it was deemed advisable to work at relatively low temperatures at all times.

Distribution between Benzene and Water—Although the details varied somewhat from time to time, the distributions all followed the same pattern. One example will be described in detail. The extract of 139 liters of urine, obtained from patients with arthritis who were receiving 17-hydroxy-11-dehydrocorticosterone, was dissolved in 400 ml. of benzene. This solution was extracted twelve times with 400 ml. of water, leaving Benzene 1. The aqueous extract was concentrated to 800 ml. and extracted seven times with 400 ml. of benzene, leaving Water 1. The benzene extract was concentrated to 300 ml. and extracted ten times with 300 ml. of water, leaving Benzene 2. The aqueous extract was concentrated to 400 ml. and extracted five times with 200 ml. of benzene, leaving Water 2. The benzene extract was concentrated to 120 ml. and extracted with 120 ml. of water, leaving Benzene 3. The final aqueous solution and the various residues were assayed for formaldehydogenic substances and qualitative tests were made for the α -ketol group (reduction of ammoniacal silver in the cold) and the α,β -unsaturated ketone group (red or orange dinitrophenylhydrazone). These tests are not highly specific but they furnished a rough guide as to which fraction or fractions contained material with the properties of an adrenal cortical hormone. The results of this particular fractionation are given in Table I. It will be noted that the "final water" was the only fraction in which a strong reduction of silver and an orange dinitrophenylhydrazone were combined. These tests indicated that only in this fraction should one expect to find appreciable amounts of an adrenal cortical hormone.

In general, the final aqueous solution was concentrated and extracted three to five times with chloroform. The solvent was removed and the residue was crystallized from chloroform or methanol. If crystallization failed at this stage, fractionation of the Girard complex was tried.

Formation of Girard's Reagent T-Ketone Complex and Its Fractional Decomposition—The residues remaining after removal of organic solvents were dissolved in 5 to 10 ml. of methanol, depending on the amount of residue, and Girard's Reagent T was added in an amount double the esti-

mated weight of the residue, followed by 2 ml. of glacial acetic acid. The mixture was allowed to stand for 24 to 48 hours at room temperature in a tightly stoppered flask. At first the acetic acid was carefully neutralized when water and ice were added to the mixture, and then a small ketonic fraction was obtained when the solution was acidified to litmus. Since this fraction was found to have no value, the procedure was changed slightly. The solution containing the Girard complex, methanol, and acetic acid was poured into a mixture of ice, water, and 6.2 ml. of 5 N sodium hydroxide. The resulting mixture, which was slightly acid to litmus, was extracted three times with chloroform and the extract was considered to be the non-ketonic fraction. The aqueous residue was then acidified progressively. A saturated solution of tartaric acid was added until Congo red paper became gray-blue, hydrochloric acid was added

TABLE I

Quantitative and Qualitative Reactions of Fractions Obtained by Benzene-Water Partitions; Extract of Urine from Patients with Arthritis

Fraction	Formaldehydogenic substances	Reduction of ammoniacal silver	Dinitrophenyl-hydrazone
	mg.		
Benzene 1.....	7.7	Weak	None
“ 2.....	3.4	None	
“ 3.....	5.9	“	
Water 1.....	55.8	Weak	Yellow
“ 2.....	24.5	“	“
Final water.....	43.3	Strong	Orange

until Congo red paper turned pure blue, and finally sufficient hydrochloric acid was added to make the concentration 1 N. After each adjustment of acidity the solution was allowed to stand at room temperature for $\frac{1}{2}$ to 1 hour and then extracted with chloroform. Sometimes another fraction was obtained by extraction after standing overnight with N hydrochloric acid. Since nothing but amorphous material was obtained from this fraction, it will not be considered further. The chloroform extracts were washed free of acid with sodium bicarbonate and water, dried, and distilled.

Melting Points—All melting points were determined with a Fisher-Johns apparatus and are recorded as read. The observed melting points of the compounds here described may vary a few degrees according to conditions. The size of crystals, rate of heating, and temperature at which the sample is placed on the heated block all influence the melting point. For the preparation of mixtures it was necessary to crush the larger crys-

tals, and the reference compound was treated in as nearly as possible the same manner as the unknown. Reference compound, unknown, and mixture were observed on the same cover-glass, which was placed on the block 15–20° below the expected melting point.

Extracts of Postoperative Urine—The extracts of the first 100 liters (Extract 1) and of the remaining 120 liters (Extract 2) of urine were worked up separately through the stage of fractionation of the Girard-ketone complex. There were some deviations from the general procedure in the treatment of Extract 1 and considerable material must have been lost, as indicated in Table II. These deviations will not be considered further since they led to poor results. Both extracts were subjected to the benzene-water partition and to the fractionation of the Girard complex. The total extract (containing 63 mg. of formaldehydogenic substances) of the urine after acidification to pH 1 was carried through the benzene-

TABLE II
Fractional Decomposition of Ketone-Girard Reagent Complex; Extracts of Postoperative Urine

Fraction	Extract 1	α,β -Un-saturated ketone	Extract 2	α,β -Un-saturated ketone
	mg.	mg.	mg.	mg.
1. Non-ketone.....			262	
2. K-1 (Congo gray).....	5		12	
3. K-2 (" blue).....	15	9	133	37
4. K-3 (N hydrochloric acid).....	11	8	94	
5. K-4 (" " " after 24 hrs.)..			15	

water partitions and then combined with Extract 2 for the Girard fractionation. Table II gives the results of the fractionation of the Girard complexes. Fraction K-2 showed an absorption maximum at 239 $m\mu$, a region in which α,β -unsaturated ketones show an absorption maximum. In the case of Extract 1 the ketonic fraction liberated with N hydrochloric acid (Fraction K-3) also showed an absorption maximum. These examinations were made with a Beckman spectrophotometer. The concentrations were 1.67, 1.10, and 5.3 mg. per cent in 95 per cent alcohol for Fractions K-2 and K-3, Extract 1 and Fraction K-2, and Extract 2, respectively; $E_{1\text{ cm.}}^{1\%} = 268, 289, \text{ and } 125$, respectively. Assuming the presence of an adrenal cortical hormone with a molecular weight of 360 and a molar extinction of 16,000, the values given in Table II for α,β -unsaturated ketone were calculated.

These three ketonic fractions were combined and diluted with water. The solution was partially distilled to remove alcohol and extracted with

chloroform. Since concentration of the chloroform solution failed to yield crystalline material, the chloroform was removed completely and the residue was dissolved in 2 ml. of pyridine and 1 ml. of acetic anhydride and allowed to stand overnight at room temperature. Dilute hydrochloric acid and ice were added and the mixture was extracted with chloroform. After the solution had been washed with sodium carbonate and dried over sodium sulfate, it was filtered and the chloroform distilled. The residue was taken up in 0.5 ml. of methanol, from which crystals separated on refrigeration overnight. They melted at 232–233° and a mixture with 17-hydroxy-11-dehydrocorticosterone (m.p. 242–243°) melted below 220°. Combination with a second crop and recrystallization gave a product which melted at 233–234°. The purified material gave no color with concentrated sulfuric acid. It reduced ammoniacal silver promptly in the cold but did not form an insoluble dinitrophenylhydrazone. The material in the mother liquor (about 200 mg.) was chromatographed on a column of 10 gm. of a 1:1 mixture of magnesium silicate and infusorial earth. Fractions were eluted with mixtures of petroleum ether and benzene, benzene, mixtures of benzene and dry ether, dry ether, and with benzene containing small concentrations of alcohol. Benzene-ether (4:1) removed 21 mg. of crystalline material which proved to be identical with the acetate which crystallized directly from the methanol solution of the crude mixture and melted at 233–234°. A second fraction of 16 mg. was eluted with benzene-ether (1:1). It melted at 245–250° after crystallization from methanol and gave a green fluorescence with concentrated sulfuric acid. A mixture with 17-hydroxy-11-dehydrocorticosterone acetate (m.p. 237–239°) melted at 225–230°. Dry ether eluted 4 mg. of material which melted at 220–222° after recrystallization from methanol and which gave a green fluorescence with sulfuric acid. A mixture with 17-hydroxycorticosterone acetate (m.p. 218–220°) melted at 218–220°. A fourth fraction, removed by 5 per cent by volume of alcohol in benzene, weighed 39 mg. and melted at 225–228° after recrystallization from methanol. It also gave the green fluorescence with sulfuric acid, reduced ammoniacal silver promptly in the cold, and formed a red dinitrophenylhydrazone.

Isolation of 17-Hydroxycorticosterone from Urine of Four Patients Receiving Adrenocorticotropic Hormone—Part of the urine was used for various determinations and part of the extracts for determination of formaldehydogenic substances. The extracts that remained were pooled in the refrigerator in 10 per cent alcoholic solution. In Cases 1 and 2 the pool of extracts assayed approximately 50 mg. of formaldehydogenic substances; in Case 3, 37 mg., and in Case 4, 26 mg. (calculated as 17-hydroxycorticosterone). In Case 1 the alcohol was removed in a vacuum and the material was subjected to distribution between benzene and water five times. The final aqueous

solution was extracted seven times with an equal volume of benzene, instead of with chloroform. After removal of the benzene the residue was transferred to a small test-tube with chloroform and this solution was concentrated to about 0.5 ml. by warming in an air stream. The crystals which separated on refrigeration overnight were collected on a filter and washed free of color with chloroform. They weighed 9.5 mg., melted at 208–210°, gave a strong green fluorescence with sulfuric acid, and reduced ammoniacal silver immediately in the cold. After recrystallization from methanol the melting point was 214–216° and a mixture with 17-hydroxycorticosterone (m.p. 214–216°) melted at 214–216°.

Approximately half of the crude material was acetylated overnight at room temperature with 4 drops of pyridine and 2 drops of acetic anhydride. Water and hydrochloric acid were added and the crude acetate was collected on a filter and washed with hydrochloric acid and water. After recrystallization from methanol the acetate melted at 214–216° and a mixture with 17-hydroxycorticosterone acetate (m.p. 216–218°) melted at 214–216°.

The material in the mother liquor from the crude 17-hydroxycorticosterone was treated with Girard's reagent and the ketone complex was decomposed fractionally. In this manner 22 mg. of non-ketone, 5 mg. of Fraction K-1 (Table II), 14 mg. of Fraction K-2, and 6 mg. of Fraction K-3 were obtained. Fraction K-2 appeared to become partly crystalline on addition of a few drops of chloroform. However, crystallization from methanol failed. Probably it was a mistake not to crystallize from a little chloroform first, since this is the best solvent yet encountered for separation of 17-hydroxycorticosterone from other urinary constituents. A few more crystals of 17-hydroxycorticosterone were obtained, after removal of the methanol, by dissolving the residue in acetone and adding petroleum ether slowly while warming. Acetylation of the remaining non-crystalline material and cautious addition of water to a methanol solution of the product gave a few crystals.

Because of what seemed to be a poor yield of 17-hydroxycorticosterone in Case 1, the extract in Case 2 was not subjected to as extensive a fractionation. After removal of alcohol the aqueous solution was diluted to 200 ml. and extracted fifteen times with 200 ml. of benzene. The benzene extract was concentrated to 150 ml. and extracted twelve times with 150 ml. of water. The aqueous extract was concentrated to 350 ml. and extracted four times with 100 ml. of chloroform. The chloroform solution was filtered, concentrated to about 3 ml., transferred to a small test-tube, and concentrated, by warming in an air stream, to about 0.5 ml. On scratching the tube, crystals began to separate and after refrigeration overnight they were collected on a filter, washed free of color with chloroform, and dried briefly at 115°. They weighed 24.3 mg., melted at 207–208°, and the other prop-

erties were the same as the crystals obtained in Case 1. After recrystallization from methanol they melted at 212–215° and a mixture with 17-hydroxycorticosterone (m.p. 214–216°) melted at 212–215°; $[\alpha]_D^{27} = +168^\circ \pm 5^\circ$ ($c = 0.310$ per cent in alcohol). The specific rotation of 17-hydroxycorticosterone has been given (12, 18) as $[\alpha]_D^{22} = +167.2^\circ \pm 2^\circ$ and $[\alpha]_D^{26} = +167^\circ \pm 3^\circ$. The acetate melted at 217–219° and a mixture with 17-hydroxycorticosterone acetate (m.p. 217–219°) melted at 217–219°.

The material in the chloroform mother liquor was acetylated but further crystalline material could not be obtained.

In Cases 3 and 4 the pools of extracts were treated essentially as in Case 2. In Case 3 the chloroform extract so obtained was concentrated to a small volume and 6.5 mg. of crystals (m.p. 203–206°) were separated. The filtrate was diluted to 10 ml. and poured onto a column of 4 gm. of a 1:1 mixture of magnesium silicate and Celite. The column was washed with a total of 250 ml. of chloroform which removed crystalline material. This material melted at 233° after recrystallization from methanol. It was identified as caffeine. A 3:1 mixture by volume of chloroform and acetone (200 ml.) removed 26 mg. of an oily fraction which was dissolved in 10 ml. of 95 per cent alcohol and filtered to remove a small amount of insoluble material. The optical rotation of this solution for sodium light was $+0.267^\circ$. This rotation corresponded to 14.8 mg. of 17-hydroxycorticosterone ($[\alpha]_D^{27} = +167^\circ$). The solution was diluted (0.3 ml. diluted to 50 ml.) and, with the use of a 1 cm. cell in a Beckman spectrophotometer, an absorption maximum was observed at 238 $m\mu$ with an extinction of 0.402. This extinction corresponded to 15.3 mg. of 17-hydroxycorticosterone ($\epsilon = 15,800$; mol. wt. = 362) in the undiluted solution. The substantial agreement of the weights calculated from the optical rotation and from the extinction at 238 $m\mu$ suggests that 17-hydroxycorticosterone was the only substance present which affected these measurements. After removal of alcohol the fraction yielded 6 mg. of crystals (m.p. 203–205°) from a small volume of chloroform. These crystals were combined with those obtained previously and recrystallized from methanol. The melting point, 212–214°, of the purified product was not depressed by admixture of 17-hydroxycorticosterone.

In Case 4 the pool (120 ml.) of extracts assayed 26 mg. of formaldehydogenic substances calculated as 17-hydroxycorticosterone. Ten extractions with 100 ml. of benzene removed 19.6 mg. The benzene solution was concentrated to 100 ml. and extracted ten times with 100 ml. of water. The combined aqueous extracts assayed 20.5 mg., indicating that all of the formaldehydogenic substances had gone from the benzene solution into the aqueous extracts. The aqueous solution was concentrated to 200 ml. and extracted four times with 50 ml. of chloroform. This extract was dried

over sodium sulfate and concentrated to 50 ml. It was then passed through a column of 3 gm. of the magnesium silicate-Celite mixture. The pattern of elution was similar to that described in Case 3. Chloroform-acetone (3:1) removed 28 mg. of material from which 7.5 mg. of crystals were obtained on crystallization from 0.5 ml. of chloroform. The crystals melted at 209–211°. After recrystallization from methanol they melted at 214–216° and a mixture with 17-hydroxycorticosterone melted at 214–216°.

The preparations obtained in Cases 3 and 4 were combined and 4.697 mg. were dissolved in 3 ml. of 95 per cent alcohol. $[\alpha]_D^{27} = +166^\circ \pm 6^\circ$. The alcohol was evaporated by warming in a stream of air and the residue was acetylated overnight at room temperature. After crystallization from methanol the product melted at 216–218° and a mixture with 17-hydroxycorticosterone acetate (m.p. 217–219°) melted at 216–218°.

Isolation of 17-Hydroxy-11-dehydrocorticosterone from Urine of Patients Receiving This Hormone and Its Acetate—Two patients with Addison's disease received a total of 2.75 gm. of 17-hydroxy-11-dehydrocorticosterone acetate. Daily extracts were made for determination of the formaldehydogenic substances and that portion (about 80 per cent) of each extract not required for this purpose was pooled in chloroform solution with others in the refrigerator. Extracts from the urine of both patients were combined. When the collection was complete, the chloroform was removed and the residue was dissolved in 100 ml. of benzene. This solution was extracted ten times with 100 ml. of water and the water solution was extracted five times with 150 ml. of chloroform. Distillation of the benzene remaining after extraction with water and of the chloroform gave 275 and 687 mg. of solids, respectively. The 687 mg. of material were treated with Girard's reagent and the ketone complex was decomposed fractionally. The non-ketonic fraction weighed 498 mg. and the ketonic fraction liberated on addition of hydrochloric acid sufficient to turn Congo red paper blue weighed 56 mg. The other ketonic fractions weighed only 10 and 20 mg. The major ketonic fraction (K-2) crystallized on addition of a few drops of chloroform. Two crops of 3.5 mg. each were obtained by crystallization from chloroform. The first crop gave a green fluorescence with sulfuric acid and an orange dinitrophenylhydrazone, reduced ammoniacal silver immediately in the cold, and melted at 216–218°. A mixture with 17-hydroxycorticosterone melted at 205–208°, whereas a mixture with 17-hydroxy-11-dehydrocorticosterone (m.p. 218–220°) melted at 216–218°. The second crop (m.p. 209–212°) was converted to the acetate which, after recrystallization twice from methanol, melted at 237–240°. A mixture with 17-hydroxy-11-dehydrocorticosterone acetate (m.p. 238–240°) melted at 238–240°.

The remainder (49 mg.) of Fraction K-2 was acetylated and, since only

a trace of crystalline material could be obtained from solvents, the product was chromatographed on a column of magnesium silicate-Celite (1:1). The same sequence of solvents was used as in the case of postoperative urine. Dry ether removed a small fraction which weighed about 1 mg. after recrystallization from methanol and which melted at 235–238°. A mixture with 17-hydroxy-11-dehydrocorticosterone acetate melted at 236–239°. Another fraction was removed with 2 per cent by volume of ethanol in benzene. It crystallized from aqueous acetone along with clumps of yellow pigment. The latter was removed by washing with methanol. The crystals melted at 244–246°. They depressed the melting point of 17-hydroxy-11-dehydrocorticosterone acetate and also the acetate melting at 245–247° obtained from postoperative urine.

The material (275 mg.) which, after extraction with water, remained in the benzene solution of the original combination of extracts, was fractionated with the aid of Girard's Reagent T. This treatment yielded 234 mg. of non-ketones and a total of 20 mg. of ketones. None of the ketonic fractions showed specific absorption in the neighborhood of 238–240 $m\mu$, and none of the fractions, including the non-ketonic, gave a detectable amount of formaldehyde on oxidation with periodic acid.

Urine was collected in varying amounts from four patients with rheumatoid arthritis who received 100 mg. per day of free 17-hydroxy-11-dehydrocorticosterone. Approximately 210 liters of urine were obtained while the patients received 19.8 gm. of the hormone during 204 patient days. Treatment of the urine and extraction were the same as in the cases of Addison's disease. The combined chloroform extracts were processed in two portions.

The results of the benzene-water partitions of the first portion are given in Table I. Similar treatment of the second portion resulted again in concentration in the final water solution of the material which reduced ammoniacal silver immediately in the cold and which gave an orange dinitrophenylhydrazone. This fraction assayed 81.3 mg. of formaldehydogenic material. This aqueous solution was combined with that obtained from the first portion (total formaldehydogenic substances of both portions 124.6 mg.), concentrated to 200 ml., and extracted seven times with 100 ml. of benzene. The solvent was removed and the residue was subjected to fractionation with the aid of Girard's reagent. The ketonic fraction (K-2) liberated by acidity sufficient to turn Congo red paper blue weighed 90 mg. and crystallized on concentration of the chloroform solution to a small volume. The chloroform was removed completely and the residue was dissolved in methanol. The solution was filtered from a small insoluble fraction, transferred to a small tube, and concentrated to about 1 ml. in an air stream while warming in a bath at 50°. Crystals

appeared only after scratching the walls of the tube. After refrigeration overnight the crystals were collected on a filter, washed with methanol, and dried at 115°. They weighed 15 mg., melted at 217–219°, and gave a green fluorescence with concentrated sulfuric acid. The methanol was removed from the filtrate and the residue was dissolved in a little acetone. Cautious addition of petroleum ether led to separation of crystals accompanied by some oil. The oil was removed by washing with 3 drops of methanol, and the remainder was recrystallized from methanol to give 6 mg. of material which melted at 225–226°. The two portions of crystalline material (21 mg.) were combined and recrystallized from methanol. This product melted at 227–229° when placed on the heated block at 215°. It still gave quite a strong green fluorescence with sulfuric acid. $[\alpha]_D^{25} = +204^\circ \pm 4^\circ$ ($c = 0.250$ per cent in alcohol). With a solution of 1 mg. per cent in 95 per cent alcohol an absorption maximum was observed at 240 $m\mu$; $\epsilon = 16,200$. Acetylation of 7 mg. with 5 drops of pyridine and 4 drops of acetic anhydride at room temperature overnight followed by crystallization of the product from methanol gave an acetate which melted at 240–242° and showed a green fluorescence with sulfuric acid. A mixture with 17-hydroxy-11-dehydrocorticosterone acetate (m.p. 240–242°) melted at 240–242°.

SUMMARY

A substance isolated as an acetate which had the properties of 17-hydroxycorticosterone acetate was obtained from the urine of patients who had undergone major surgical operations. The amount was too small to permit certain identification. 17-Hydroxycorticosterone was isolated in significant amounts from the urine of four patients suffering from rheumatoid arthritis who received adrenocorticotrophic hormone in doses of 100 mg. per day. The urine of similar patients who received 100 mg. per day of 17-hydroxy-11-dehydrocorticosterone yielded small amounts of this hormone unchanged. Small amounts of 17-hydroxy-11-dehydrocorticosterone also were isolated from the urine of two patients suffering from Addison's disease who were treated with the acetate of this substance. There was some evidence of a physiologic reduction of the 11-keto group of 17-hydroxy-11-dehydrocorticosterone to a hydroxyl group. Methods are described for isolation of these adrenal cortical hormones from urine.

BIBLIOGRAPHY

1. Venning, E. H., and Kazmin, V., *Endocrinology*, **39**, 131 (1946).
2. Dorfman, R. I., Horwitt, B. N., and Fish, W. R., *Science*, **96**, 496 (1942).
3. Horwitt, B. N., and Dorfman, R. I., *Science*, **97**, 337 (1943).
4. Schiller, S., and Dorfman, R. I., *Endocrinology*, **33**, 402 (1943).
5. Shipley, R. A., Dorfman, R. I., and Horwitt, B. N., *Am. J. Physiol.*, **139**, 742 (1943).

6. Perla, D., and Gottesman, J. M., *Proc. Soc. Exp. Biol. and Med.*, **28**, 1024 (1931).
7. Weil, P., and Browne, J. S. L., *Science*, **90**, 445 (1939).
8. Talbot, N. B., Saltzman, A. H., Wixom, R. L., and Wolfe, J. K., *J. Biol. Chem.*, **160**, 535 (1945).
9. Heard, R. D. H., and Sobel, H., *J. Biol. Chem.*, **165**, 687 (1946).
10. Corcoran, A. C., and Page, I. H., *J. Lab. and Clin. Med.*, **33**, 1326 (1948).
11. Daughaday, W. H., Jaffe, H., and Williams, R. H., *J. Clin. Endocrinol.*, **8**, 166 (1948).
12. Mason, H. L., and Sprague, R. G., *J. Biol. Chem.*, **175**, 451 (1948).
13. Hench, P. S., Kendall, E. C., Slocumb, C. H., and Polley, H. F., *Proc. Staff Meetings Mayo Clin.*, **24**, 18 (1949).
14. Girard, A., and Sandulesco, G., *Helv. chim. acta*, **19**, 1095 (1936).
15. Reichstein, T., *Helv. chim. acta*, **19**, 1107 (1936).
16. Reichstein, T., and Shoppee, C. W., in Harris, R. S., and Thimann, K. V., *Vitamins and hormones*, New York, **1**, 345 (1943).
17. Wintersteiner, O., and Pfiffner, J. J., *J. Biol. Chem.*, **116**, 291 (1936).
18. Reichstein, T., *Helv. chim. acta*, **20**, 953 (1937).

A NOTE ON THE FAILURE OF NON-ISOTOPIC URANIUM TO INHIBIT RESORPTIVE PROCESSES IN THE RAT FEMUR*

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In an earlier study (1), uranyl nitrate (1.5 to 3.0 mg. per kilo) enriched with the α -emitter U^{233} (15 μ c. per kilo) was administered intraperitoneally to young rats to permit a simultaneous radioautographic and microscopic study of ground sections of femur. It was observed that the resorptive processes in the bones of these animals were inhibited. 40 days after injection, the "new," active metaphysis, practically devoid of radioactivity, appeared to be forming and resorbing normally. Parts of the "old" metaphysis, however, impregnated with the enriched uranium at the time of injection, remained unresorbed in the marrow cavity. Also, some of the marrow surface of the shaft had not been resorbed, while new bone continued to be deposited on the periosteal surface, resulting in a thickening of the diaphyseal wall.

Uranium has been demonstrated to reduce the solubility of tooth enamel (2, 3), and it was suggested (1), therefore, that the uranium-impregnated bone was resistant to resorption because of its lower solubility.

This hypothesis was tested by the administration of non-isotopic uranium to young rats. In these animals, no disturbance of resorption characteristic of that seen previously was observed.

EXPERIMENTAL

Fifteen rats weighing 30 gm. were injected intraperitoneally with non-isotopic $UO_2(NO_3)_2 \cdot 6H_2O$. In two groups of five rats each a tolerance was established (4) by eight small doses (0.15 mg. of U per kilo) administered every 2 or 3 days. The final large dose was 2 mg. of U per kilo for five of these animals and 3 mg. of U per kilo for the other five. Three rats received 1.5 mg. of U per kilo every 10 days, and two others 1.8 mg. per kilo every 10 days until progressive loss of weight was observed. The total dose of uranium injected in all cases was larger than that containing U^{233} given in the previous experiments. Three of the animals in the last two groups died after three, four, and five doses respectively. At weekly

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intervals for 10 weeks, each experimental rat along with five control rats was anesthetized lightly with ether and roentgenograms were made of the two hind legs.

Results

Careful study of the roentgenograms revealed no inhibition of the resorptive processes. A reduction in the growth rate of these animals as a result of the injections was accompanied by a temporary reduction in the growth of the femur and a narrowing of the metaphyseal zone. However, as body growth resumed, no thickening of the shaft walls or unresorbed metaphyses could be detected. Roentgenograms were made for periods of from 4 to 9 weeks after the last uranium injection. The animals were sacrificed from 13 to 16 weeks after the last injection. All long bones were cut longitudinally. The mineral material was stained according to the copper acetate-picric acid method described by Harz (5) and examined microscopically for evidence of disturbed resorption. The experimental bones were the same as those of the controls.

It must be concluded that the inhibition of the resorptive processes observed previously was not caused by any specific chemical effect of the uranium itself but rather by radiation. Recently Bloom (6) has reported such an inhibition in bone following the administration of several radioactive "bone-seeking" elements. As described by Bloom, radiation doses of the same order of magnitude administered to growing rats resulted in changes in the bone which were almost identical with those obtained with our radioactive uranium preparation (1).

SUMMARY

In an earlier study, it was observed that the resorptive processes were inhibited in the bones of rats receiving an injection of radioactive uranyl nitrate. From results obtained with animals receiving non-isotopic uranyl nitrate, it must be concluded that the inhibition of the resorptive processes observed previously was not caused by any specific chemical effect of the uranium itself but rather by radiation.

The authors wish to express their appreciation of the assistance of Mrs. Florence Van Slyke in making the roentgenograms.

BIBLIOGRAPHY

1. Neuman, M. W., and Neuman, W. F., *J. Biol. Chem.*, **175**, 711 (1948).
2. Bibby, B. G., and McKinnon, I., *J. Dent. Res.*, **25**, 161 (1946).
3. Muhler, J. C., and Van Huysen, G., *J. Dent. Res.*, **26**, 119 (1947).
4. Haven, F. L., and Randall, C., *J. Biol. Chem.*, **175**, 737 (1948).
5. Harz, P. H., *Am. J. Clin. Med.*, **17**, 750 (1947).
6. Bloom, W., *Histopathology of irradiation from external and internal sources*, New York, chapter 5 (1948).

STUDIES ON THE METABOLISM OF PHOTOSYNTHETIC BACTERIA

V. PHOTOPRODUCTION OF HYDROGEN AND NITROGEN FIXATION BY RHODOSPIRILLUM RUBRUM*

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The photosynthetic purple bacteria can utilize a variety of organic compounds anaerobically in the light (5, 6). This "photodecomposition" provides both the energy and materials for growth of the organisms. Although certain substrates can also be metabolized by resting cells of purple bacteria under anaerobic conditions in the dark (7, 8), the bacteria cannot grow at the expense of these processes. The inability to develop in the dark in the absence of oxygen is one of the characteristics which distinguish purple bacteria from typical heterotrophic anaerobes.

Details of the mechanism of the light-stimulated attack on organic substrates have not yet been obtained. From data describing the over-all gas metabolism in certain cases, it is possible to devise several alternative mechanisms to account for the results observed. These include (a) complete decomposition of the substrate to CO_2 and "hydrogen," followed by reduction of some or all of the CO_2 to the carbohydrate level, and (b) decomposition of a portion of the substrate, etc., as above, together with assimilation of the remainder. The latter mechanism appears to be the more probable, but a definitive proof by means of various isotopically labeled substrates is still required.

In connection with sequence (a) it has been long known from the work of Gaffron (9) and Roelofsen (10, 11) that H_2 can serve as a hydrogen donor for the photoreduction of CO_2 by purple bacteria and that certain photosynthetic bacteria can even grow on the basis of this reaction. These fundamental experiments demonstrate that purple bacteria can reduce CO_2 under certain circumstances. However, the existence of this reaction obviously does not prove that the carbon of complex organic substrates is available to the organisms only after complete oxidation to CO_2 .

* For Papers I to IV see Gest and Kamen (1-4).

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Recent experiments have shown that the non-sulfur purple bacterium, *Rhodospirillum rubrum*, produces molecular hydrogen in addition to CO_2 during anaerobic growth in certain media containing oxidized organic compounds (2, 3) and also that this organism is able to fix molecular nitrogen (4). The present paper deals mainly with the photoproduction of H_2 by resting cell suspensions of *R. rubrum*. Further observations on the fixation of N_2 and its relation to H_2 formation are also recorded. On the basis of the results described, a tentative mechanism for the light-stimulated formation of H_2 is presented.

EXPERIMENTAL

Preparation of Cells—Pure cultures of *Rhodospirillum rubrum* (strain SI) were grown, unless otherwise noted, in a medium of the following composition: *dl*-malic acid, 3.5 gm.; L-glutamic acid, 4 gm.; sodium citrate \cdot $5\frac{1}{2}\text{H}_2\text{O}$, 0.8 gm.; biotin, 5 γ ; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 gm.; CaCl_2 , 38 mg.; KH_2PO_4 , 120 mg.; K_2HPO_4 , 180 mg.; Difco yeast extract, 250 mg.; distilled water, 1 liter; pH adjusted to 7 with NaOH before autoclaving. The medium was sterilized in a reagent bottle and seeded with a generous inoculum from a stab culture in 1 to 2 per cent agar plus 0.3 per cent Difco yeast extract.¹ Additional sterile medium was used to fill the bottle completely and the bottle closed with a flamed glass stopper. Anaerobic cultures prepared in this manner were incubated at approximately 30° with good illumination (incandescent lamps) and shaken once a day to disperse sedimented bacteria. Under these conditions excellent growth accompanied by the production of CO_2 and H_2 was usually obtained in 3 to 5 days.

The bacteria from such cultures were harvested by centrifugation, washed twice with 0.05 M phosphate buffer (pH 7.2) containing 0.02 per cent $\text{NaS} \cdot 9\text{H}_2\text{O}$, and suspended in 0.05 M phosphate buffer at pH 6.6 for use in manometric experiments. A rough estimate of the wet cell volume in the final suspension was obtained by centrifugation of an aliquot in a calibrated Hopkins vaccine tube for 30 to 45 minutes at about 2600 R.P.M.

Manometric Measurements—The production or consumption of H_2 was followed manometrically at 30° in Warburg vessels provided with 0.2 ml. of 10 per cent KOH in the center well. Proof that the alkali-insoluble gas produced was H_2 was obtained by use of additional vessels containing a hydrogen absorber composed of about 75 mg. of palladized asbestos (prepared according to the directions of Treadwell and Hall (12)) mixed with 0.2 ml. of saturated methylene blue solution (13). The palladized asbestos-methylene blue mixture was found to be effective in absorbing H_2 in control experiments. With an argon or helium atmosphere no pressure

¹ Freshly prepared medium of this composition was inoculated and incubated in the light to provide stock stab cultures.

changes occurred in experimental vessels containing both H_2 and CO_2 absorbers. Accordingly, H_2 and CO_2 are the only gases which appear to be involved in the metabolism under anaerobic conditions.

A rough indication of simultaneous CO_2 production was given by pressure changes in flasks containing no absorbers. The actual CO_2 production was not and probably cannot be quantitatively determined with certainty because of the occurrence of photoreduction, *i.e.* photoconsumption of CO_2 with H_2 . In addition to this complicating reaction, it is probable that the presence of alkali (or an H_2 absorber) in the system may well influence the extent of H_2 (or CO_2) production. For these reasons, many of the data presented below must be considered as qualitative.

The gas atmospheres used are indicated in connection with the particular experiments. In all cases, added substrates were neutralized to approximately pH 7. Photosynthetic metabolism was measured during illumination of the vessels by a bank of 60 watt incandescent lamps placed above the bath. Metabolic activity in the dark was followed in vessels wrapped with aluminum foil or by covering the entire bath with a dark cloth.

Results

Metabolism of Rhodospirillum rubrum (SI) under Nitrogen Atmosphere—Initial attempts to observe H_2 production manometrically from substrates effective in growing cultures (2, 3) were made in a nitrogen atmosphere. H_2 formation could not be detected in the dark or light in any instance. Variation of pH and addition of inorganic activators, boiled juices of *R. rubrum*, or reducing agents such as cysteine had no effect. The lack of H_2 production under N_2 could not be ascribed to an oxyhydrogen reaction occurring at the expense of O_2 contamination, because the inclusion of yellow phosphorus or alkaline pyrogallol in the vessels did not influence the results. Furthermore, analysis of the N_2 used by the alkaline pyrogallol method disclosed a relatively small quantity of O_2 (<0.3 per cent).

Although no H_2 is formed by resting cells under N_2 , CO_2 is produced photochemically when substrates such as malate are furnished. These observations suggest that N_2 is not an inert gas with respect to H_2 evolution by *R. rubrum* (SI). Experimental results described below corroborate this conclusion.

With certain substrates, liberation of H_2 may occur under an N_2 atmosphere. The only clear cut example we have observed with *R. rubrum* is the decomposition of formate to CO_2 and H_2 . If the bacteria are grown in the medium already described, the cells will decompose formate in the dark at a moderately good rate only after a prolonged adaptation period.²

² If formate is added to the growth medium, the cells obtained can decompose formate in the dark almost immediately.

Simultaneous measurements in the light, however, show comparatively small pressure changes even after long periods. This is apparently due to the fact that the organisms can reutilize the dark products (H_2 and CO_2) in a light-induced reaction. The recombination of CO_2 and H_2 in the light can be directly observed simply by illuminating vessels in which *R. rubrum* has been allowed to decompose formate in the dark. Immediate gas uptake is observed. The balance between gas production and consumption during illumination can sometimes be disturbed by placing absorbers for CO_2 or H_2 in the vessel, in which case greater positive pressures for one or the other component gas are observed in the light. These results are similar to those reported by Nakamura (7) for the decomposition of formate and glucose in light and dark by another non-sulfur purple bacterium, *Rhodobacillus palustris*.

It is evident that quantitative interpretation of manometric data obtained during illumination is very difficult in certain cases because of the photochemical reduction of CO_2 by H_2 . The lack of any significant manometric changes does not necessarily mean low metabolic activity.

In summary, it appears that various strains of both sulfur and non-sulfur purple bacteria are able to decompose certain compounds such as formate, glucose, glycerol, glycerophosphate, pyruvate, or endogenous reserves in the dark under N_2 with the production of H_2 (7, 8, 11). On the other hand, certain oxidized substrates such as malate are not decomposed under N_2 in the dark by other strains or species; they are only attacked in the light with the production of excess CO_2 .

Photoproduction of Hydrogen from Malate—Under a gas phase of argon or helium, washed cells of *R. rubrum* (SI) metabolize malate in the light, with the production of CO_2 and H_2 . Typical results illustrating the course of H_2 evolution under helium are given in Fig. 1, which clearly demonstrates the photochemical activation of the reaction.

Although CO_2 evolution occurs almost immediately under these circumstances, H_2 does not appear in significant amounts for 15 to 60 minutes. The extent of the lag apparently depends on the particular bacterial suspension used. The cause of this delay in H_2 formation is not yet known: It is obviously not attributable to adaptive enzyme formation because malate was present in the growth medium and because CO_2 is produced from this substrate almost immediately. It is possible that the lag represents the time required for reduction of an enzyme system or saturation of hydrogen acceptors, but it seems equally likely that it is related to a requirement for soluble cofactors which are lost during washing of the cells.

The production of H_2 does not occur in the absence of substrate and, within limits, its initial rate depends on the concentration of malate.

Variation of the initial pH between 5.8 and 7.5 does not markedly affect the rate when substrate is not limiting. In addition, preliminary experiments indicate that both the *d* and *l* isomers of malic acid are utilized.

R. rubrum (SI) also produces H_2 from malate at a rapid rate in the light under a gas phase of 100 per cent H_2 (see Fig. 5). This is surprising in view of the fact that excess H_2 normally suppresses H_2 formation in bacterial fermentations (13-16). It is of interest that H_2 evolution is not particularly sensitive to the presence of small concentrations of O_2 as can be seen from Fig. 2. The bacteria were placed under an atmosphere of 2.5 per cent O_2 in helium and allowed to oxidize malate for 3.5 hours in the

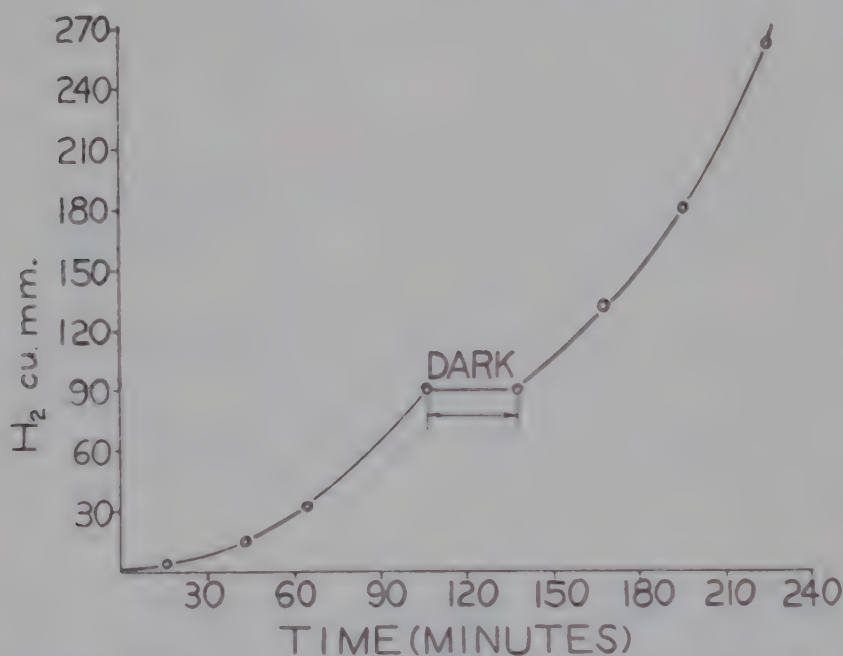


FIG. 1. Photoproduction of H_2 by *Rhodospirillum rubrum* (SI). 50 c.mm. of washed cells were suspended in 2 ml. of 0.05 M phosphate buffer (pH 6.6). 5 mg. of *dl*-malate tipped in at zero time. The center well contained 0.2 ml. of 10 per cent KOH; 100 per cent He in the gas space.

dark, at which time a considerable amount of O_2 still remained in the vessel. Upon illumination, H_2 was immediately produced, as shown in Fig. 2.

Specificity of Photoproduction of Hydrogen—A number of biochemically significant compounds were tested as H_2 producers with resting cells in 0.05 M phosphate buffer (pH 6.6) under a helium atmosphere with alkali in the center well. After thermal equilibration in the dark, the substrate was tipped in and the Warburg vessels illuminated as already described. Control vessels with malate as substrate were always included to be sure that the organisms were metabolically active as far as H_2 evolution was concerned. The results of this survey are listed in Table I.

The evolution of H_2 from malate, oxalacetate, fumarate, and pyruvate under these conditions is dependent on illumination. With the three

effective dicarboxylic acids, the rates of H₂ production are approximately the same for equal concentrations of substrate and there is the usual lag period before H₂ appears.

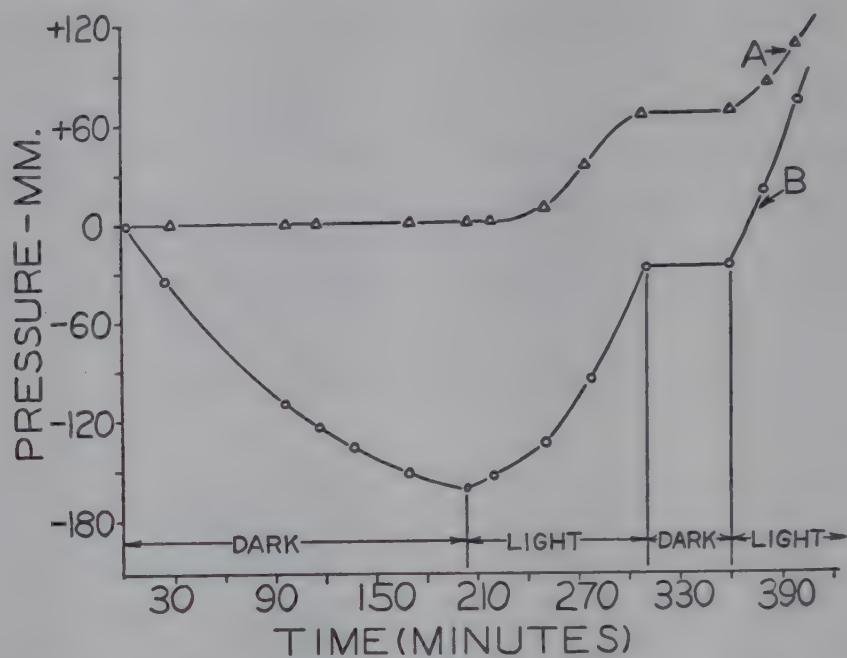


FIG. 2. Photoproduction of H₂ from malate by *Rhodospirillum rubrum* (SI) in the presence of oxygen. The vessels contained 80 c.mm. of washed cells suspended in 2 ml. of 0.05 M phosphate buffer (pH 6.6). 5 mg. of *dl*-malate tipped in at zero time; 0.2 ml. of 10 per cent KOH in center well. Curve A, 100 per cent He; Curve B, 2.5 per cent O₂-97.5 per cent He.

TABLE I

Specificity of Photoproduction of Hydrogen by Rhodospirillum rubrum (SI)

Washed bacteria were suspended in 0.05 M phosphate buffer (pH 6.6) as usual; 0.2 ml. of 10 per cent KOH in center well; gas phase, 100 per cent He; 20 to 40 μM of neutralized substrate added.

Hydrogen produced	No hydrogen produced		
Malate	Glucose	Uracil	Acetate
Oxalacetate	Glucose-1-phosphate	Glycine	Butyrate
Fumarate	Fructose	Alanine	Tartrate
Pyruvate*	Mannose	Aspartate	Succinate*
	Inositol	Glutamate*	α-Ketoglutarate
	Ribose	Formate	Citrate
	Lactate	Ethanol	Isocitrate
	Glycerophosphate	Glycolate	cis Aconitate

* See the text.

In comparison with the dicarboxylic acids, pyruvate shows only feeble H₂ production. The H₂ evolution from malate, oxalacetate, and fumarate exceeds 1 mole per mole of substrate added. It has been found that about

0.1 mole of H_2 is produced for each mole of pyruvate utilized by resting cell suspensions in the presence of alkali. Quantitative measurements of the CO_2 produced (in the absence of alkali) and of the substrate disappearing indicate that about 80 per cent of the pyruvate carbon is directly assimilated. The formation of H_2 from pyruvate by resting cells of *R. rubrum* is not appreciably inhibited by an atmosphere of 100 per cent H_2 .

Succinate is decomposed photochemically by resting cells of *R. rubrum* (SI) as shown by CO_2 evolution, but H_2 is not observed as a product. Attempts to induce H_2 formation from succinate alone by variation of pH, substrate concentration, etc., were unsuccessful. This result was unexpected in view of the fact that H_2 is found in anaerobic cultures of *R.*

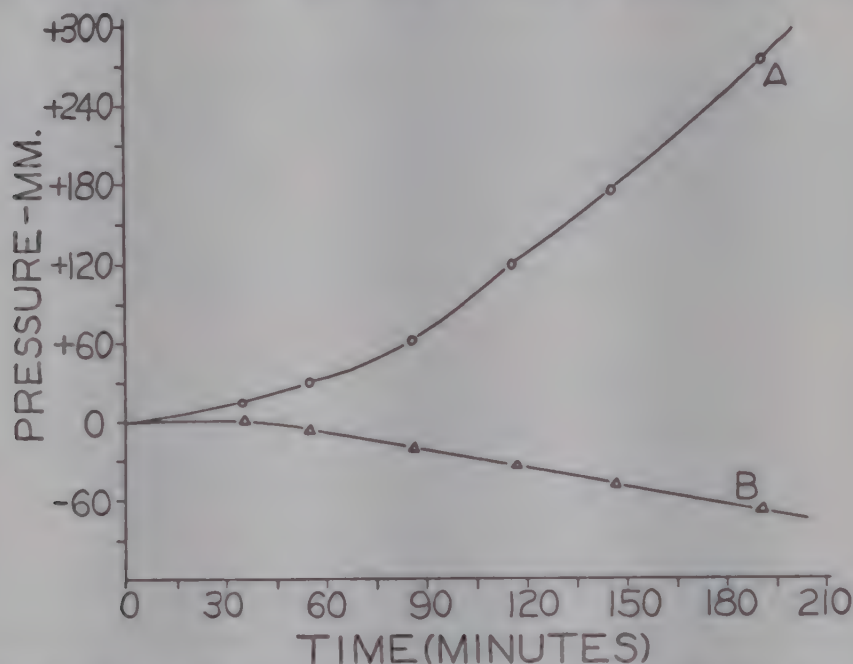


FIG. 3. Inhibition of photoproduction of H_2 from malate by N_2 . 60 c.mm. of washed cells suspended in 2 ml. of 0.05 M phosphate buffer (pH 6.6). 5 mg. of *dl*-malate tipped in at zero time; 0.2 ml. of 10 per cent KOH in center well. Curve A, 100 per cent He; Curve B, 100 per cent N_2 .

rubrum (SI) growing in a succinate + glutamate medium (2, 3). As indicated in Table I, glutamate also does not serve as a hydrogen source. If both glutamate and succinate are added to resting cells under helium, however, H_2 production is observed after a lag period varying from 1 to 5 hours. Although this observation is consistent with the results noted in growing cultures, the explanation for this complex effect is not obvious at the present time.

Inhibition of H_2 Production by N_2 and NH_4Cl —The inhibitory effect of N_2 on photoproduction of H_2 from malate is shown by the experimental results given in Fig. 3. Under a gas phase of helium with KOH in the system, a vigorous production of H_2 is observed; under N_2 , gas is taken up. Although a small amount of O_2 was present in the N_2 in this instance, the

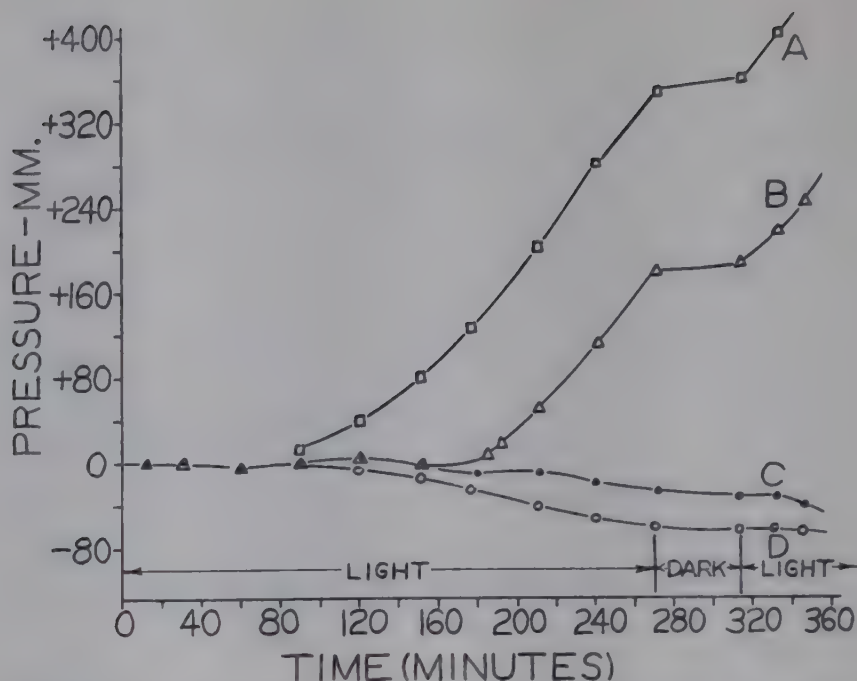


FIG. 4. Inhibition of photoproduction of H_2 from malate by N_2 ; reactivation with He. 72 c.mm. of washed cells suspended in 2 ml. of 0.05 M phosphate buffer (pH 6.6). 5 mg. of *dl*-malate tipped in at zero time; 0.2 ml. of 10 per cent KOH in center well; 0.2 ml. of alkaline pyrogallol in side arm. Curve A, 100 per cent He; Curve B, 20 per cent N_2 + 80 per cent He until + 180 minutes, then 100 per cent He; Curve C, 20 per cent N_2 + 80 per cent He; Curve D, 100 per cent N_2 .

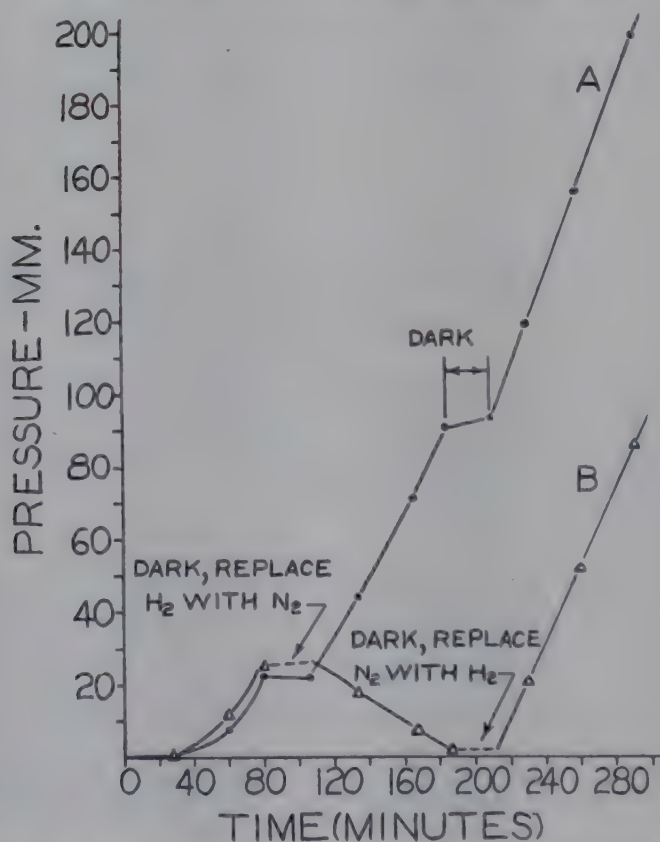


FIG. 5. Inhibition of photoproduction of H_2 from malate by N_2 ; reactivation with H_2 . 60 c.mm. of washed cells suspended in 2 ml. of 0.05 M phosphate buffer (pH 6.6). 5 mg. of *dl*-malate tipped in at zero time; 0.2 ml. of 10 per cent KOH in center well. In Curve A, the gas phase was 100 per cent H_2 throughout the experiment; in Curve B, the vessel contained 100 per cent H_2 initially and the atmosphere was changed as indicated.

uptake was in all probability a consumption of N_2 , because similar results are noted when alkaline pyrogallol is included in the vessel (see Fig. 4).

From Fig. 4, it is evident that 20 per cent N_2 in the gas phase is sufficient to inhibit H_2 evolution. Furthermore, the effect of N_2 is reversible as indicated in Curve B, where replacement of the 20 per cent $N_2 + 80$ per cent He atmosphere by 100 per cent He after 3 hours led to immediate production of H_2 .

If the bacteria are exposed to N_2 after a period of hydrogen production (under H_2), no further evolution of H_2 is observed. Subsequent replace-

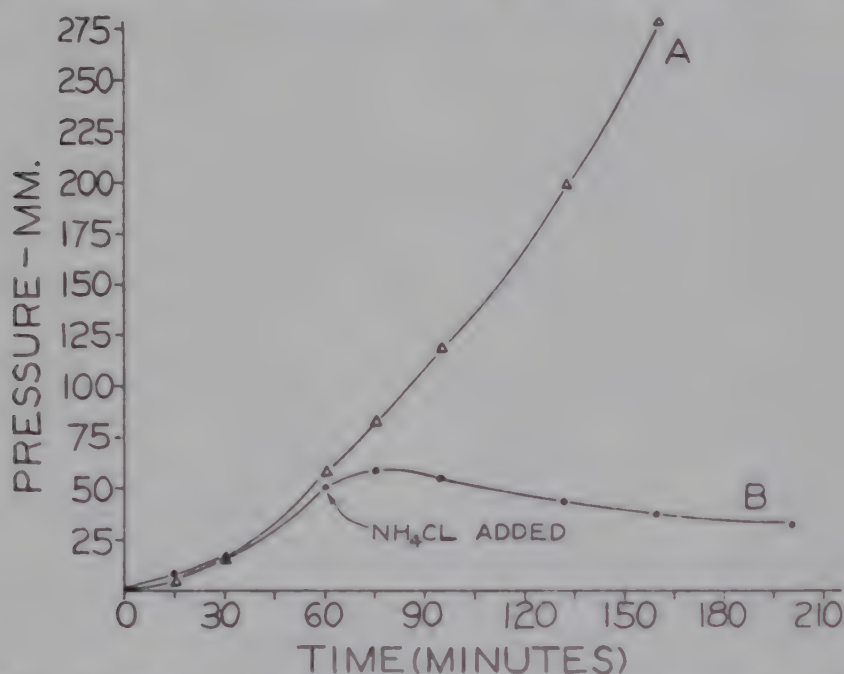


FIG. 6. Effect of NH_4Cl on photoproduction of H_2 from malate by *Rhodospirillum rubrum* (SI). 60 c.mm. of washed cells were suspended in 2 ml. of 0.05 M phosphate buffer (pH 6.6). 5 mg. of *dl*-malate tipped in at zero time; 0.2 ml. of 10 per cent KOH in center well; 100 per cent He in the gas space. At 60 minutes, 1 mg. of NH_4Cl was tipped into the vessel in Curve B. Curve A shows photoproduction of H_2 in controls (no NH_4Cl added).

ment of the N_2 atmosphere with 100 per cent H_2 leads to an immediate resumption of H_2 production (Fig. 5). All of these results show clearly that molecular nitrogen is not an inert gas with respect to photochemical H_2 evolution and are consistent with the notion that a fixation or exchange of N_2 can occur in these organisms and that this is responsible for the inhibition noted. Direct evidence that N_2 fixation occurs is given in the next section.

One of the most probable products of biological N_2 fixation is ammonia, and the latter might consequently also be expected to inhibit the production of H_2 by *R. rubrum*. That this is the case can be seen from Fig. 6, which shows the effect of adding NH_4Cl to a suspension of *R. rubrum* (SI) producing H_2 under an atmosphere of helium. This result is in accord

with observations on the effect of NH_4Cl on H_2 production by growing cultures of the same organism (3). Although NH_4Cl is an excellent nitrogen source for *R. rubrum* (SI), it inhibits the formation of H_2 when added to the growth medium in amounts of 0.5 gm. per liter or more.

Addition of NH_4Cl affects gas production under an atmosphere of 100 per cent H_2 similarly. In this case, however, NH_4Cl also causes a large gas uptake when no absorbers are present in the vessel. The details of these experiments are not included here, owing to the fact that under a gas phase of H_2 interpretation of the results is complicated by the occurrence of photoreduction and possibly other types of H_2 uptake. In addition to photoreduction of CO_2 with H_2 , dark H_2 consumption with other acceptors may also occur (8, 17, 18).

It is probable that N_2 influences other reactions in addition to photoproduction of H_2 . Previous manometric experiments with *Rhodospirillum* (and other purple bacteria) in which N_2 was employed as an inert gas may have to be reexamined with other gas mixtures in view of the data presented here.

Experiments on Nitrogen Fixation—The observation that photoproduction of hydrogen gas was repressed by molecular nitrogen as well as by ammonia suggested the existence in *R. rubrum* of a nitrogenase system, hitherto unsuspected. Typical experiments establishing *R. rubrum* as a nitrogen-fixing organism will be described in this section.

Hydrogen-producing bacteria grown anaerobically in the standard malate medium for 4 days were washed and suspended in 38 ml. of a medium with the following composition: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mg.; 0.05 M phosphate buffer of pH 6.6, 10 ml.; *dl*-malic acid, 0.35 gm.; neutralized to pH 7.1 with 6 N NaOH and diluted with distilled H_2O to 100 ml. 10 ml. aliquots were placed in each of two Warburg vessels (capacity, 70 ml.). Another 10 ml. aliquot was boiled in a water bath for 10 minutes to provide a dead cell control and then put into a third Warburg vessel. Each vessel was then fortified with 0.2 ml. of biotin solution (2 γ) and 0.1 ml. of a solution containing trace amounts of Mn, B, Cu, Zn, and Mo. The center wells of the two vessels containing live organisms were provided with a filter paper soaked with 0.4 ml. of 6 N NaOH for absorption of CO_2 arising during the test period. Each vessel contained about 300 c.mm. wet volume of cells corresponding to about 2 mg. of Kjeldahl N.

All three vessels were placed on a vacuum line, including a manometer and a nitrogen-generating system. The vacuum line was evacuated to about 0.1 atmosphere and filled with helium to atmospheric pressure. This procedure was repeated three times, so that residual air contamination was rendered negligible. After the final pumping, N^{15} -labeled molecular nitrogen was generated from Eastman Kodak $\text{N}^{15}\text{H}_4\text{NO}_3$ (30 atom

per cent excess N^{15}) by adding NaOBr solution dropwise to the dry ammonium salt. The evolved nitrogen was passed slowly through two liquid air traps, and then into the Warburg vessels. The total N_2 pressure reached a final value of 0.1 atmosphere (7.5 cm. of Hg). The pressure was brought to atmospheric by addition of 0.9 atmosphere of helium. One vessel containing live organisms was wrapped in aluminum foil, thereby being kept dark. The vessels were then closed and placed in an illuminated bath at 30° for 120 hours.

At the end of the test run, the cells were separated by centrifugation, washed, transferred to Kjeldahl flasks, and dried overnight in a vacuum desiccator. Kjeldahl analyses were made and the resultant samples submitted to isotopic analysis. The sample maintained in the light was found to contain 3.14 atom per cent excess; the dark sample contained 0.18₇ atom per cent excess and the controls 0.00₈ atom per cent excess. These results showed the presence of a nitrogenase system, apparently

TABLE II
Fixation of N^{15} by Rhodospirillum rubrum (SI)

Sample	N^{15} content
	atom per cent excess
Supernatant liquid. No NH_4Cl	3.02
“ “ With NH_4Cl	0.09 ₉
Residual cells. No NH_4Cl	2.19
“ “ With NH_4Cl	0.09 ₄
Control, boiled cells.....	0.05 ₉

photoactivated. The possibility that small amounts of labeled ammonia were responsible for the results obtained was eliminated by analysis of the evolved nitrogen which revealed no detectable ammonia ($<2 \times 10^{-3}$ mg. of NH_3 in a total of 6 mg. as N_2).

In another experiment, the same medium was used but with the addition in one vessel of 5 mg. of NH_4Cl per 10 ml. of suspension. In this run, all vessels were maintained under illumination. The experiment lasted 10 days, at which time some autolysis was noted. The cell suspensions were divided into supernatant liquid and residual cells and analyzed for N and isotopic content. The results are given in Table II. These results indicate clearly the reality of the fixation and its expected inhibition by ammonia.

To ascertain that the incorporation of isotopic nitrogen did not merely represent exchange between molecular and cell nitrogen, growth experiments involving the use of molecular nitrogen as the major source of nitrogen were set up. In an exploratory experiment, a malate-mineral salt

medium free of all nitrogenous compounds except for a few micrograms of biotin per liter was inoculated with a dilute suspension of bacterial cells and exposed to light in a closed bottle under anaerobic conditions for several weeks. A similar control bottle was kept at 0° in the dark. A marked growth of organisms occurred in the experimental bottle apparently at the expense of the air nitrogen dissolved in the culture fluid. Kjeldahl analysis revealed no nitrogen (<0.005 mg. of N) per 10 ml. of the control, while the experimental bottle showed 0.56 mg. of N per 10 ml. of cell suspension.

Another experiment was performed by use of the same culture medium fortified with yeast extract. 200 ml. of this medium in a 500 ml. culture flask were inoculated with a dilute suspension of bacterial cells. The gas space was filled with a mixture of N₂ and H₂ and the flask closed under sterile conditions. A control flask was maintained at 0° in the dark. The Kjeldahl N of the control assayed 0.25 mg. per 10 ml. of suspension. After 30 days, good growth was noted in the experimental flask. The Kjeldahl N content of the suspension in the experimental vessel had increased to 1.63 mg. per 10 ml. of suspension (analysis in triplicate). Thus, a 7-fold increase in cellular nitrogen had occurred at the expense of the molecular nitrogen, providing unambiguous proof for nitrogen fixation.

DISCUSSION

The results of these researches fall into two categories, one dealing with hydrogen transfer during photoassimilation and the other with occurrence of a nitrogen fixation apparatus in photosynthetic systems. In the first category, it has been demonstrated that under certain conditions the normal transfer of hydrogen in the photometabolic chain may be shunted through a hydrogenase system so that molecular hydrogen appears as a primary product of photosynthesis. In attempting to assess the significance of this phenomenon it is necessary to recall briefly the experience of other investigators with non-sulfur purple bacteria. Gaffron (9, 19) observed that these organisms could accomplish a photoassimilation of CO₂ provided various organic substrates or hydrogen were present. Gaffron, Nakamura *et al.*, and, in particular, van Niel showed that the hydrogen activating and transferring systems of these organisms possess great versatility (7, 8, 17, 18, 20). It is surprising therefore to find that, despite the presence of large amounts of CO₂, suspensions of *R. rubrum* produce under illumination amounts of free hydrogen nearly equivalent on a molar basis to the CO₂ evolved. Present theories on the photosynthetic process do not envisage the possibility that molecular hydrogen will be encountered as a major photosynthetic product.

To account for the hydrogen production it may be assumed as a working hypothesis that, during metabolism of the organic substrate, metabolic

fragments are formed which can undergo direct assimilation into cellular material and that under these conditions CO_2 fixation is not obligatory. This is to say that total oxidation of substrate to CO_2 is not required as a step in assimilation. According to a line of reasoning used often by Gaf-fron (21) the anaerobic photoassimilation of CO_2 in the presence of malic acid can be pictured in the following manner: the first step involves the photofission of H_2O in the presence of intermediates X and Y to produce equivalent amounts of a reducing system HX and an oxidizing system YOH , as shown in equation (1).



It has been observed in this laboratory that approximately 50 per cent of the malate carbon appears in the cells at the carbohydrate level, while

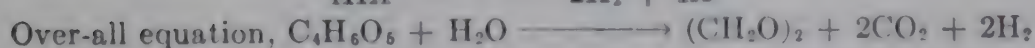
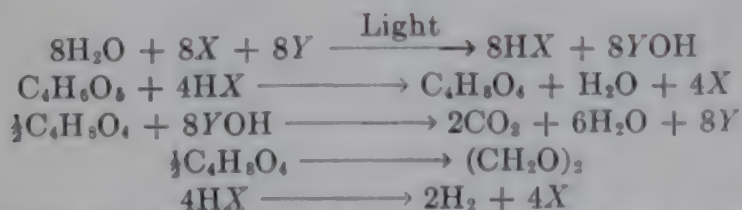
TABLE III

Yield of Molecular Hydrogen Expected for Varying Assimilation of Pyruvate Carbon

Assimilation	H_2 evolved per mole pyruvate
	moles
0.67	1.00
0.80	0.20
0.82	0.08
0.85	-0.10
1.00	-1.00

1.00 = complete assimilation.

half is oxidized to CO_2 and H_2O , which may be represented in a variety of ways relative to reactions involving HX and YOH . One way is shown in the following reaction scheme.



With pyruvate as substrate a much lower yield of molecular hydrogen is observed. Assuming again that all cellular material is at the level of carbohydrate, values for H_2 evolution can be calculated, as shown in Table III, with various amounts of assimilation.

Resting cells have been observed in these researches to assimilate approximately 80 per cent of pyruvate carbon with a net evolution of about 0.1 mole of H_2 per mole of pyruvate disappearing.

Another reaction mechanism perhaps more expressive of the assimilation reactions involves individual treatment of the carbon atoms of the malate molecule. One may suppose that the two carboxyl groups are oxidized by 2 YOH molecules to CO_2 and water. The $-\text{CHOH}$ carbon, being already at the carbohydrate level, may undergo direct assimilation, while the methylene carbon ($-\text{CH}_2$) requires 2 YOH molecules to undergo oxidation to the carbohydrate level. On this basis the 2 central (reduced) carbon atoms of malic acid are the 2 carbons assimilated, while the 2 carboxyl carbons are the source of CO_2 . In the process 4 YOH molecules are used up, leaving an excess of 4 HX molecules.

There is no inkling at present as to why the excess HX is liberated as H_2 rather than being used to reduce CO_2 . A perusal of the list of substrates given in Table I throws little light on this question. Of the many substrates tested, only a few which happen to possess an over-all oxidation level slightly higher than cell material (carbohydrate) are effective in evoking hydrogen. However, one can find many examples in which there is an over-all oxidation level apparently favorable for H_2 production but in which no evolution of H_2 is observed.

It is evident that a real understanding of hydrogen production requires more detailed knowledge of the assimilation mechanism than is available at present. The present observations emphasize the rôle of organic substrates more as carbon than as hydrogen donors. It is implied that hydrogen which appears as molecular hydrogen is not derived by simple dehydrogenation of the substrate but rather indirectly with water as the ultimate hydrogen donor. A direct test of this hypothesis would involve a demonstration of the source of hydrogen produced. Experiments with use of substrates labeled with isotopic hydrogen would yield a positive answer provided no label appeared in the photohydrogen. Appearance of label in the photohydrogen could arise from a rapid exchange of hydrogen atoms in the dicarboxylic acid with the hydrogen atoms of water. This would render ambiguous an interpretation of the appearance of labeled molecular H_2 . Nevertheless it appears worth while to attempt such an experiment.³

These considerations relating to the photohydrogen evolution by *Rhodospirillum* recall ideas previously advanced by Gaffron who, indeed, mentioned the possibility of such a phenomenon in purple bacteria ((16) p. 239). This worker in collaboration with Rubin was able to demonstrate

³ A. San Pietro and D. Rittenberg (private communication) have found that in the absence of substrate and light *R. rubrum* (SI) fails to effect an exchange between D_2O and H_2 . This shows apparently that the hydrogenase of these organisms differs materially from those commonly found in *Bacterium coli* and *Proteus vulgaris* which do not require active metabolism to effect rapid exchange.

a photochemical evolution of hydrogen, distinct from fermentative hydrogen production, with use of the green alga, *Scenedesmus*. However, photo-production of H_2 by *Scenedesmus* differs radically in the following respects from that reported here with *Rhodospirillum*: (1) It is not inhibited by nitrogen and is repressed by hydrogen. (2) It is dependent on intracellular hydrogen donors. (3) It is evoked only after long incubation under anaerobic conditions and is repressed at normal photosynthetic light intensities unless an inhibitor like dinitrophenol is present. (4) The amount of hydrogen produced in *Scenedesmus* is lower by almost two orders of magnitude. Thus, Gaffron and Rubin (16) observed about $2\ \mu M$ of H_2 produced by 100 c.mm. (wet volume) of cells in 12 hours. A typical yield of H_2 by use of *Rhodospirillum* with malate substrate is about $120\ \mu M$ for the same quantity of cells and for the same time. It should be emphasized that the photohydrogen evolution in *Rhodospirillum* is observed under normal growth conditions. The many dissimilarities cited would appear to justify the position that the photohydrogen production in *Rhodospirillum* is unique and distinct from the only instance previously cited of photohydrogen production, that in *Scenedesmus*.

The present observations provide a direct demonstration of hydrogenase activity during photosynthetic hydrogen transfer. The possibility of realizing this phenomenon in previous researches with Athiorhodaceae has been denied, because the nitrogen source used in growth experiments was ammonia and the "inert" gas used in resting cell experiments was usually nitrogen. The recent development of synthetic media in which glutamic acid as well as other amino acids could be used to supply the nitrogen requirements in place of ammonia (22, 23) provided the necessary background for elaborating conditions under which photohydrogen production could be observed.

These remarks lead to a discussion of the unexpected finding that *R. rubrum* is an active free growing nitrogen-fixing organism. While only one strain of *R. rubrum* has been tested so far for this property it is quite likely that other strains and species of Athiorhodaceae will show similar abilities. It has been observed that most strains of *R. rubrum* will produce photohydrogen as does *R. rubrum* (SI) (3); hence it would not be surprising if they are also found to be nitrogen-fixing organisms.

The mechanism for nitrogen fixation in *R. rubrum* appears to be connected intimately with the hydrogenase activity of these organisms. Lee and Wilson (24) observed that in *Azotobacter*, the classical non-photosynthetic free growing nitrogen-fixing organism, hydrogenase activity was inhibited by combined nitrogen. However, no such inhibition could be established in *Azotobacter* with use of molecular nitrogen. The present observation of the inhibition of H_2 production by molecular nitrogen in

R. rubrum appears to supply this link between N_2 and the hydrogen transfer systems of nitrogen fixation.

In regard to the actual mechanism of the nitrogen or ammonia inhibition of photohydrogen production, it may be only guessed at present that an obligatory reductive amination is responsible. Thus, H_2 is evolved in growing cultures when glutamic acid is the nitrogen source, presumably because protein formation can take place without net incorporation of N_2 or ammonia. On this basis the presence of free ammonia or nitrogen would be considered as providing a substrate for combination with the excess hydrogen from the photosynthetically produced HX , thereby effectively suppressing hydrogen formation. Extension of the present observations to other amino acids is now being undertaken.

It is obvious that the comparative biochemistry of nitrogen fixation will require reexamination in the light of these results. No observation seems to be recorded relating to a photosensitized nitrogen fixation up to the time of this research. Blue-green algae have been found to exhibit some nitrogen-fixing capacity (25-27), but no direct demonstration of the necessity for light has been reported. It remains to be seen whether *R. rubrum* actually requires photosynthetic conditions for nitrogen fixation. No experiments on aerobic fixation in the dark have yet been attempted. The possibility that many nitrogen-fixing organisms have been overlooked because of inappropriate culture conditions also looms large, as may be readily appreciated from the circumstances involved in the discovery of the fixation by *R. rubrum*.

Further progress in elucidating the hydrogen transfer mechanisms of *R. rubrum* both in photosynthesis and in nitrogen fixation would appear to depend on success in preparing cell-free extracts which can be used to demonstrate partial reactions. The prospects appear to be encouraging. It has already been shown that it is possible to prepare cell-free extracts of *R. rubrum* exhibiting good malic acid dehydrogenase activity. Dr. A. Mehler, working in our laboratory, found that a thick paste of cells, ground with glass at 0° in neutral bicarbonate buffer, could be centrifuged to give an intensely purple-colored supernatant liquid which could reduce oxalacetate, by the use of diphosphopyridine nucleotide as H donor, with an activity comparing favorably with the best preparations reported by Ochoa and his collaborators using pig heart.⁴ The same preparation showed no lactic dehydrogenase activity, mirroring the behavior of intact

⁴ S. Korkes has confirmed these observations. The significance of the observation that intact cells do not attack certain tricarboxylic acid cycle intermediates cannot be judged at present. The highly preferential evocation of hydrogen by the 4-carbon dicarboxylic acids is reminiscent of the malate-pyruvate system proposed by Ochoa for CO_2 fixation.

cells which also fail to attack lactate, or, for that matter, any typical carbohydrate.

The metabolic characteristics of the Athiorhodaceae suggest that these organisms may provide test systems more amenable than the green algae have been so far for elucidating mechanisms of photoassimilation.

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SUMMARY

1. Experiments described in this and preceding papers demonstrate photochemical evolution of hydrogen when the non-sulfur purple bacterium, *Rhodospirillum rubrum* (strain SI), is grown in a synthetic medium containing a dicarboxylic acid, glutamic acid, mineral salts, and biotin. Growing cultures evolve hydrogen when the dicarboxylic acid is malic, fumaric, or succinic acid.

2. Resting cells will produce photohydrogen only from malic, fumaric, oxalacetic, and pyruvic acids out of some thirty substrates tested, including fatty acids, amino acids, sugars, alcohols, etc. Succinic acid appears to be exceptional in that glutamic acid is required in conjunction with it for photohydrogen production.

3. The hydrogen production does not appear to occur via intermediary production of formate followed by decomposition of formate, nor does phosphorylysis of pyruvate seem to be involved.

4. Among other unique properties, the photoproduction of hydrogen is found to be repressed either by ammonium ion or by molecular nitrogen. This phenomenon has led to the discovery of a nitrogen-fixing system in these photosynthetic bacteria. The implications of these results for the comparative biochemistry of photosynthesis and nitrogen fixation are discussed.

Addendum—In a recent communication Lindstrom, Burris, and Wilson (28) have presented data confirming the present findings on *R. rubrum* and extending observations to representatives of the sulfur photosynthetic bacteria which they show also to be capable of N₂ fixation. In addition preliminary data are presented indicating that N₂ fixation in *R. rubrum*

is associated with the light anaerobic metabolism rather than with a dark aerobic process.

BIBLIOGRAPHY

1. Gest, H., and Kamen, M. D., *J. Biol. Chem.*, **176**, 299 (1948).
2. Gest, H., and Kamen, M. D., *Science*, **109**, 558 (1949).
3. Gest, H., and Kamen, M. D., *J. Bact.*, **58**, 239 (1949).
4. Kamen, M. D., and Gest, H., *Science*, **109**, 560 (1949).
5. van Niel, C. B., *Bact. Rev.*, **8**, 1 (1944).
6. Muller, F. M., *Arch. Mikrobiol.*, **4**, 131 (1933).
7. Nakamura, H., *Acta Phytochim.*, **10**, 211 (1938).
8. Nakamura, H., *Acta Phytochim.*, **11**, 109 (1939).
9. Gaffron, H., *Biochem. Z.*, **275**, 301 (1935).
10. Roelofsen, P. A., *Proc. Roy. Acad. Sc. Amsterdam*, **37**, 660 (1934).
11. Roelofsen, P. A., On photosynthesis of the Thiorhodaceae, Dissertation, Utrecht (1935).
12. Treadwell, F. P., and Hall, W. T., *Analytical chemistry; Quantitative analysis*, New York, 7th edition (1928).
13. Cardon, B. P., and Barker, H. A., *Arch. Biochem.*, **12**, 165 (1947).
14. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **158**, 505 (1945).
15. Kubowitz, F., *Biochem. Z.*, **274**, 285 (1934).
16. Gaffron, H., and Rubin, J., *J. Gen. Physiol.*, **26**, 219 (1942).
17. Nakamura, H., *Acta Phytochim.*, **10**, 259 (1938).
18. Yamagata, S., and Nakamura, H., *Acta Phytochim.*, **10**, 297 (1938).
19. Gaffron, H., *Biochem. Z.*, **260**, 1 (1933).
20. van Niel, C. B., *Advances in Enzymol.*, **1**, 263 (1941).
21. Gaffron, H., *Biol. Rev. Cambridge Phil. Soc.*, **19**, 1 (1944).
22. Hutner, S. H., *Arch. Biochem.*, **3**, 439 (1943-44).
23. Hutner, S. H., *J. Bact.*, **52**, 213 (1946).
24. Lee, S. B., and Wilson, P. W., *J. Biol. Chem.*, **151**, 377 (1943).
25. Drewes, K., *Centr. Bakt.*, **76**, 88 (1928).
26. Allison, F. E., and Morris, H. J., *Science*, **71**, 221 (1930).
27. Burris, R. H., Eppling, F. J., Wahlin, H. B., and Wilson, P. W., *Proc. Soil Sc. Soc. America*, **7**, 258 (1942).
28. Lindstrom, E. S., Burris, R. H., and Wilson, P. W., *J. Bact.*, **58**, 313 (1949).

PRODUCTION OF RADIOACTIVE SERUM ALBUMIN BY LIVER SLICES*

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Evidence that the formation of serum albumin occurs in the liver has been based mainly on the observation that a decline of the circulating albumin level occurs following hepatectomy, Eck fistula, or during cirrhosis (1). It has also been found that incorporation of S^{35} -labeled methionine into serum albumin decreases upon hepatectomy (2). From such findings, *in vivo*, one cannot conclude whether the liver forms serum albumin unaided, or whether it contributes but one link in a process involving both hepatic and non-hepatic tissues.

In a previous study (3), the incorporation of radioactive $C^{14}O_2$ into the dicarboxylic amino acids of the proteins of rabbit liver slices was observed. This incorporation has now been examined in greater detail in slices of chicken livers, which exhibit 2 to 3 times the turnover rates previously obtained. In the course of this study it was found that a highly radioactive protein having physical, immunological, and chemical properties indistinguishable from those of chicken serum albumin appeared in the incubating medium.

EXPERIMENTAL

Incubation—Non-fasted chickens, 6 to 12 weeks old, were killed by decapitation and the livers cut into 0.5 mm. slices with a Stadie slicer. Approximately 10 gm. of slices were washed free of loose cells and debris by swirling in a flask containing incubation medium ($Ca = 20$, $Na = 72$, $K = 73$, $Cl = 125$, $HCO_3 = 40$ m.eq. per liter; pH 7.5), rinsed, and placed in a 500 cc. Erlenmeyer flask containing 50 cc. of the same medium.

After the vessel had been flushed with 5 per cent CO_2 -95 per cent O_2 , 0.01 to 0.02 mm of $Na_2C^{14}O_3$ (2.7×10^8 counts per minute per mm), was added and the flask securely stoppered. The presence or absence of pyruvate did not affect the degree of incorporation, sufficient energy apparently being available from the stores of utilizable substrate within the slices.

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The vessels were rocked for 3 hours at 38°, after which the slices were removed and rinsed in fresh medium. Loose cells and débris were removed from the combined medium and washings by centrifugation and the supernatant was subjected to alcohol fractionation as described below.

Preparation of Samples—Inorganic CO₂ samples were prepared from the gas phase of each flask by precipitation as barium carbonate. Samples taken at the end of incubation usually showed a 10 to 15 per cent lower specific activity than did the initial samples. Calculation of incorporation was based on the final CO₂ specific activity, which ranged from 500,000 to 1,500,000 counts per minute per mm of CO₂.

Protein samples were obtained by homogenizing the slices with cold 5 per cent trichloroacetic acid. Samples of the various protein precipitates were suspended in the same reagent. The trichloroacetic acid precipitates were washed three times with 5 per cent trichloroacetic acid solution, three times with warm 50 per cent alcohol-ether, and once with warm ether. The proteins were hydrolyzed by autoclaving at 15 pounds per sq. in. with 6 N HCl for 16 hours.

The hydrolyzed amino acids were decarboxylated with ninhydrin (4), and the liberated CO₂ precipitated as barium carbonate. This barium carbonate and that from the gas samples were washed and counted as previously described (5), by use of a counter with a background of 5 to 6 counts per minute. Appropriate self-absorption corrections were applied, and all counts were adjusted on the basis of a standard BaC¹⁴O₃ sample to compensate for variations in counter sensitivity. Duration of counting was such as to make the computed probable error less than 2 per cent. Most samples gave 50 to 1000 counts per minute.

Results

In preliminary experiments the distribution of radioactivity in the various insoluble granular portions and in crude fractions of the soluble liver proteins was investigated. The data in Table I demonstrate that, of the fractions studied, only the proteins which had entered the medium during incubation showed significantly greater specific radioactivity than did the liver slice itself. The similarity of nuclear and cytoplasmic specific activities agrees with the findings of Bergstrand *et al.* (6) with N¹⁵-labeled glycine.

Alcohol Fractionation—To determine whether specific protein components in the medium were responsible for its higher radioactivity, the proteins of the centrifuged solution were subjected to low temperature alcohol fractionation. In order to obtain a solution of low ionic strength and to remove most of the HC¹⁴O₃⁻, the bulk of the proteins was precipitated at an alcohol concentration of 50 per cent, pH 6.5, temperature

-18° . Alcohol was slowly added to the medium, maintaining the temperature near the freezing point of the solution, and the precipitate was centrifuged at -18° after storage overnight. This precipitate was dissolved in water at 0° , and the pH readjusted to 6.5 with 2 M acetate buffer of pH 5. The ionic strength of this solution was about 0.02 and the protein concentration about 0.2 per cent. Various alcohol fractions were then precipitated and removed consecutively by centrifugation at 8000 to 10,000 R.P.M. at temperatures from 0° to -15° , and their specific radioactivities determined. The results of a trial fractionation are presented in Table II.

In a series of such experiments (Table III) the highest radioactivity was consistently found in the protein obtained between 33 per cent and

TABLE I
Relative Radioactivity of Liver Cell Fractions

Fraction	Method of preparation	Specific activity ratio*
		$\frac{\text{Fraction proteins}}{\text{Slice proteins}}$
Nuclei	Hogeboom (7)	0.83
"	5% citric acid	0.97
Mitochondria	Hogeboom (7)	1.00
Ribonucleoprotein	Griffin (8)	0.97
" "	Schneider (9)	1.13
Soluble residue, pH 5		0.99
Insoluble "		1.14
Incubation medium including cellular debris		2.3
Cellular debris		1.03

* Counts made on CO_2 liberated by ninhydrin from washed, hydrolyzed protein.

43 per cent alcohol. It seemed possible that a single protein species was responsible for the high specific activity of this fraction, especially since the amount obtained constituted only about 3 per cent of the total protein in the medium, or about 3 mg. per 10 gm. of slices. The alcohol solubility suggested that it might be an albumin, and to test the possibility that it represented serum albumin the following experiments were undertaken.

Immunological Data—Antiserum was produced in two rabbits by injecting twelve gradually increasing doses of 2 to 4 mg. of whole chick serum protein over a 4 week period (10). The antigen was prepared by dialyzing fresh chicken serum against saline solution, followed by filtration through an ultrafine fritted glass filter. The antiserum showed a titer of 1:100,000 and gave a precipitin reaction against the sera of several

TABLE II
Example of Alcohol Fractionation of Proteins of Medium

Fraction	Specific activity ratio* $\frac{\text{Fraction proteins}}{\text{Slice proteins}}$
Total medium.....	3.9
0-50% alcohol ppt. from medium.....	4.0
50% alcohol supernatant.....	2.9
0-50% fraction redissolved	
Insoluble in water at pH 6.5.....	3.1
0-5% ppt.....	4.4
5-10% ppt.....	4.0
10-15% ".....	4.2
15-20% ".....	5.1
20-25% ".....	5.4
25-33% ".....	5.1
33-42% ".....	11.7
42% supernatant.....	4.7

* Counts made on CO₂ liberated by ninhydrin from washed, hydrolyzed protein

TABLE III
Results of Alcohol Fractionations

Experi- ment No.	Specific activity of slice proteins*	Specific activity ratio* $\frac{\text{Medium proteins}}{\text{Slice proteins}}$	Fraction with highest specific activity	
			Alcohol fraction	Specific activity ratio* $\frac{\text{Fraction proteins}}{\text{Slice proteins}}$
14	4480	2.3 (cellular débris included)		
15	2640	7.8 (centrifuged clear)		
16	6400		23% supernatant	9.1
17	1030†	3.8 " "	28-41% ppt.	8.8
			41% supernatant	8.8
18	885†		33-45% ppt.	8.1
19	2140	4.3 " "	35-43% "	16.5
20	3940	3.9 " "	33-42% "	11.7
21	5460	3.8 " "		
	4230	4.0 " "		
25	2890		33-43% ppt.	11.5-12
26	3560		33-43% "	11-12
23	Labeled alanine as substrate	4.1 " "	33-43% "	8.4

* Counts per minute per mm made on CO₂ liberated by ninhydrin from washed, hydrolyzed protein; corrected to 10⁶ counts per minute per mm in inorganic CO₂.
† 20 gm. slices per flask; in other experiments, 10 gm. slices per flask.

breeds of chickens, and no reaction against dog serum. A precipitate was obtained with chicken serum albumin isolated by alcohol fractionation, demonstrating the antigenic activity of this protein.

The antiserum was used to test both the clear centrifuged medium and the 33 to 43 per cent alcohol fraction (redissolved in saline) from two experiments. The results (Table IV) show that 80 to 90 per cent of the total counts in the active fraction was precipitated. Since the antiserum had been prepared against whole chicken serum, it is possible that some of the counts precipitated from the total medium represented serum globulins. However, globulins would not be expected to be present in the 33 to 43 per cent alcohol fraction.

Ultracentrifugation—The 33 to 43 per cent alcohol fraction from one of the later experiments was dialyzed against phosphate buffer and subjected to ultracentrifugation. For comparison, chicken serum albumin,

TABLE IV
*Antiserum Precipitations**

Radioactivity precipitated from total medium	Radioactivity precipitated from 33-43 per cent alcohol fraction	Radioactivity not precipitated from 33-43 per cent alcohol fraction
7,520	1720	170
15,600	3200	800

* Total counts per minute of CO₂ liberated by ninhydrin from washed, hydrolyzed protein.

freed of most of the globulins by one precipitation in 50 per cent saturated ammonium sulfate, was analyzed under nearly identical conditions. The results (Table V) reveal that the incubation medium fraction is 83 per cent pure with respect to a component having a sedimentation constant equal to that of serum albumin within the limits of the determination.

That the radioactivity must be due mainly to the major component is suggested by the following consideration. In a typical 3 hour incubation, the specific activity of the carboxyl carbon of the entire 33 to 43 per cent fraction was 4.1 per cent of the inorganic CO₂ specific activity. If all the radioactivity resided in the minor components, which comprised 17 per cent of the fraction, the specific activity of their carboxyl carbons would be $100/17 \times 4.1$ per cent or 24 per cent of that of the inorganic CO₂.

About 25 per cent of the CO₂ released by ninhydrin from a liver protein hydrolysate is derived from glutamic and aspartic acids. Since these two amino acids appear to be responsible for the bulk of the radioactivity incorporated into amino acid carboxyl groups from CO₂ (3), the

dicarboxylic amino acid carboxyl carbons of the minor components would have a specific activity equal to $100/25 \times 24$ per cent or 96 per cent of that of the inorganic CO_2 . This seems unlikely in 3 hours of incubation.

Electrophoresis—Since the amount of protein obtained in the 33 to 43 per cent alcohol fraction was insufficient for direct electrophoretic examination, 10 mg. of the fraction were mixed with about 90 mg. of chicken serum proteins, and the mixture examined in a five compartment Klett-Tiselius electrophoresis apparatus, after dialysis against 0.055 M phosphate buffer of pH 7.7. Mobilities were not calculated since compensation was employed to separate the albumin peak from the globulin peaks, thus producing an albumin sample on the ascending side and a globulin sample on the descending side.

Radioactivity determinations made on these two samples (Table VI)

TABLE V
Ultracentrifugation Results

Sample analyzed	Concentration	pH	Ionic strength	$s_{20,w}$ (Svedberg units) and per cent of total		
	<i>per cent</i>					
33-43% alcohol fraction	0.618	7.7	0.15	1.77 (4.0%)	4.39 (83.3%)	6.91 (12.8%)
Chicken serum albumin	0.750	7.7	0.15		4.33 (92.6%)	6.68 (7.4%)

show that the specific activity of the albumin component was 47 times that of the globulin components.

Miscellaneous Data—The absorption spectra of the incubation medium fraction and of chicken serum albumin isolated by alcohol fractionation agreed closely in the range 250 to 300 $m\mu$.

As a check on the nature of the incorporated C^{14} , counts were made on the CO_2 evolved specifically from the α -carboxyl carbon of glutamic acid, with squash glutamic decarboxylase (11). In this experiment, non-radioactive serum albumin was added as a diluent after isolation of the 33 to 43 per cent alcohol fraction in order to obtain sufficient material for the decarboxylase reaction. The CO_2 obtained in this manner showed a specific activity 2.9 times that of the average amino acid carboxyl carbon as evolved by reaction with ninhydrin. On the basis of the glutamic and aspartic acid contents of serum albumin (12), and assuming that all of the carboxyl radioactivity resides, equally distributed, in the α -carboxyl of glutamic acid and both carboxyls of aspartic acid (3), the specific activity ratio is calculated to be 2.86 for human and 3.00 for bovine serum albumin. Figures for chicken serum albumin are not available at present.

To determine whether amino acids other than glutamic and aspartic acids are incorporated into the fraction isolated, an experiment was performed to test the incorporation of carboxyl-labeled alanine. The last line in Table III shows that the ratio of the specific activity of the 33 to 43 per cent alcohol fraction to that of the slices was about the same as in the experiments with $C^{14}O_2$. Correction was made for possible incorporation of $C^{14}O_2$ resulting from alanine oxidation, based on the final specific activity of the inorganic CO_2 .

An experiment to study time relations showed that, after a 30 minute lag phase, the degree of incorporation of radioactivity from $C^{14}O_2$ into the "serum albumin" of the medium proceeded linearly for at least 4 hours. Incorporation into the proteins of the slice showed a lag phase of only 5 minutes.

TABLE VI
Electrophoresis of Radioactive Fraction with Chicken Serum

Sample	Specific activity
	<i>counts per min. per ml*</i>
Albumin	10600
Globulins	224

* Counts made on CO_2 liberated by ninhydrin from washed, hydrolyzed protein.

The observed protein is apparently produced by the slice but not retained in it to any extent. Alcohol fractionation of the homogenized slice protein was performed concurrently with that of the medium proteins in one experiment, and, while the 33 to 43 per cent alcohol fraction of the medium was 16.5 times the specific activity of the total slice, the corresponding fraction of the slice itself was only 0.94 times that specific activity. Whether the serum albumin is formed at the cell membrane or inside the cell and rapidly secreted is a subject for further study.

DISCUSSION

The data above indicate that chicken liver slices incorporate dicarboxylic amino acids and alanine into serum albumin and release this albumin into the surrounding medium. On the basis of radioactivity incorporated, this serum albumin was the most rapidly formed of any fraction isolated from the slices or the medium, a finding which correlates with the high turnover rates of serum albumin found *in vivo* (13, 14). The specific activity of the glutamic acid α -carboxyl carbon of the albumin reached 12 per cent of the inorganic CO_2 specific activity in the average experiment.

The CO₂-bicarbonate incubation medium was believed to represent physiological conditions closely, except for the high potassium and calcium concentration, which had been found necessary for glycogen synthesis in slices (15), and except for the omission of oxidizable substrate. However, even the fasting animal has been found capable of producing plasma proteins (1). The high rate of incorporation of radioactivity adds supporting evidence to the suggestion of Madden and Whipple (1, 16) and of Northrop (17) that the liver may function as protein former for many other tissues of the body, relieving more specialized tissues of the task of forming basic protein units by secreting plasma proteins utilizable in smaller or larger fragments for protein synthesis. Some basis for such a hypothesis is found in the demonstration by Daft *et al.* (18) and Terry *et al.* (19) of maintenance of nitrogen balance in fasting dogs by injection of serum.

SUMMARY

1. Liver cell fractions were prepared following incubation of chicken liver slices in C¹⁴O₂-bicarbonate medium. None showed a significantly higher rate of incorporation of dicarboxylic amino acids into protein than did the average slice protein.

2. Low temperature alcohol fractionation of the proteins which entered the medium during incubation yielded a fraction having 8 to 16 times the specific activity of the slice.

3. Immunological, ultracentrifugal, and electrophoretic tests indicated that this fraction consisted mainly of a protein indistinguishable from serum albumin. This lends confirmation to earlier suggestions that the liver is the site of serum albumin formation.

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BIBLIOGRAPHY

1. Madden, S. C., and Whipple, G. H., *Physiol. Rev.*, **20**, 194 (1940).
2. Tarver, H., and Reinhardt, W. O., *J. Biol. Chem.*, **167**, 395 (1947).
3. Anfinsen, C. B., Beloff, A., and Solomon, A. K., *J. Biol. Chem.*, **179**, 1001 (1949).
4. Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141**, 671 (1941).
5. Solomon, A. K., Gould, R. G., and Anfinsen, C. B., *Phys. Rev.*, **72**, 1097 (1947).
6. Bergstrand, A., Eliasson, N. A., Hammarsten, E., Norberg, B., Reichard, P., and von Ubisch, H., *Cold Spring Harbor Symposia Quant. Biol.*, **13**, 22 (1948).
7. Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., *J. Biol. Chem.*, **172**, 619 (1948).

8. Griffin, A. C., Nye, W. N., Noda, L., and Luck, J. M., *J. Biol. Chem.*, **176**, 1225 (1948).
9. Schneider, W. C., *J. Biol. Chem.*, **166**, 595 (1946).
10. Kabat, E. A., and Mayer, M. M., *Experimental immunochemistry*, Springfield (1948).
11. Schales, O., Mims, V., and Schales, S. S., *Arch. Biochem.*, **10**, 455 (1946).
12. Brand, E., *Ann. New York Acad. Sc.*, **47**, 187 (1946).
13. Schoenheimer, R., Ratner, S., Rittenberg, D., and Heidelberger, M., *J. Biol. Chem.*, **144**, 541 (1942).
14. Wu, H., and Rittenberg, D., *J. Biol. Chem.*, **179**, 847 (1949).
15. Hastings, A. B., Solomon, A. K., Anfinsen, C. B., Gould, R. G., and Rosenberg, I. N., *J. Biol. Chem.*, **177**, 717 (1949).
16. Madden, S. C. and Whipple, G. H., *Am. J. Med. Sc.*, **211**, 149 (1946).
17. Northrop, J. H., in Parpart, A. K., *The chemistry and physiology of growth*, Princeton (1949).
18. Daft, F. S., Robschey-Robbins, F. S., and Whipple, G. H., *J. Biol. Chem.*, **123**, 87 (1938).
19. Terry, R., Sandrock, W. E., Nye, R. E., and Whipple, G. H., *J. Exp. Med.*, **87**, 547 (1948).

DIPHOSPHOPYRIDINE NUCLEOTIDE IN THE INACTIVATION OF α -ESTRADIOL BY RAT LIVER*

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The rapid disappearance of estrogen which has been injected into animals has been traced to inactivation by the liver (1). Estrogen from an ovary transplanted into the mesentery (2), from pellets implanted intrasplenically (3-6), or from solutions injected intrasplenically (7-9) has been shown to be inactivated by passage through the liver prior to entrance into the systemic circulation. Other evidence has been furnished by experiments on animals whose livers had been altered by chemical damage (10, 11) or by partial hepatectomy (12, 13).

In 1934, Zondek (1) was able to demonstrate the inactivation of estrone by five of seven preparations of rat liver mince *in vitro*. Other writers have demonstrated estrogen inactivation by rat liver slices (14-16), but comparatively little success has been obtained with broken cell preparations or liver extracts (17, 18). In this laboratory, the ability of rat liver mince to inactivate α -estradiol has been found to be less than that of slices taken from the identical liver. Evidence that this difference is due to the destruction in mince of an essential factor, diphosphopyridine nucleotide (cozymase, coenzyme I; hereafter referred to as DPN), has been presented in a preliminary report (19), and has recently been confirmed by DeMeio *et al.* (20). Further work on the rôle of DPN in inactivation of estrogen by liver is now presented.

Methods

The livers used in these studies were obtained from 200 to 300 gm. male rats (Maguran Farms), which had been maintained on a diet of Purina laboratory chow. The animals were decapitated and the livers removed immediately. Supported by a glass slide, the liver was sliced with a

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razor blade. The wet weight of each slice was taken at once and the tissue then dropped into a 10 ml. flask containing 1.5 γ of α -estradiol in 5 ml. of normal saline (0.9 per cent NaCl solution), buffered to pH 7.4 with M/75 phosphate buffer. A total of 250 (± 2) mg. of liver tissue was added to each flask; thus the mixture contained 50 mg. of liver for each 0.3 γ of α -estradiol.

Mince was prepared by dropping a portion of the same liver from which slices had been cut into a chilled Waring blender containing cold saline, and blending for 3 minutes. The proportion of 1 gm. of liver per 30 ml. of saline was found to produce the most homogeneous blend. The homogenate was usually allowed to stand for 10 minutes at 7° before measuring the samples in order to get rid of most of the air bubbles. 10 ml. of this mixture, representing 333 mg. of liver, were added to a 50 ml. flask containing 2 γ of α -estradiol in 10 ml. of buffered saline solution (pH 7.4); the resulting phosphate concentration was M/75. This mixture thus contained 50 mg. of liver per 0.3 γ of α -estradiol, as did the preparation with slices. The differences in total volume of solutions of slices and of mince were found to have no effect upon the degree of inactivation. All solutions were incubated aerobically with shaking for 1 hour at 38°. This was found necessary for inactivation of α -estradiol. Following incubation, slices were ground in a glass homogenizer, and each original 5 ml. of solution quantitatively diluted to 15 ml. with saline. All solutions were then kept at 7° during the assay period. Suspensions which were placed in boiling water to stop enzymic activity gave the same results as did those which were placed in the refrigerator after incubation.

All mixtures were assayed for estrogenic activity by the uterine weight response of 24 to 26 day-old female rats (Maguran Farms), according to the method of Lauson *et al.* (21). Injections of 0.5 ml. of the whole mixture were made twice daily for 3 days; each animal thus received a total dose of 0.3 γ of α -estradiol (or its metabolites) and 50 mg. of liver. Experiments were carried out in groups of 60 to 100 rats; three or four animals were used for each preparation assayed. Within each group the uterine weight response was fairly uniform at each assay level, but was somewhat variable from experiment to experiment. A linear relationship was found between the logarithm of the amount of α -estradiol injected and the logarithm of the uterine weight obtained. If this line is extended to the logarithm of the uterine weight of uninjected controls, it is found that this control weight corresponds to about 0.01 γ of α -estradiol. Since 0.01 γ of α -estradiol was taken as the control uterine weight in all experiments, it was possible to express the results in terms of microgram equivalents of α -estradiol. When the data of Lauson *et al.* (21) are plotted in this manner, essentially the same relationship is found.

In each experiment a control set of uninjected rats and a set receiving

0.3 γ of α -estradiol were included. The logarithms of the average uterine weights of these sets were plotted against the logarithms of the α -estradiol (assuming 0.01 γ for the uninjected animals). From the straight line drawn between these two points the α -estradiol equivalents of the assay animals were interpolated. Intermediate standards gave results which fell close to this level. The values obtained for 0.2 γ of α -estradiol standards read from this line were within 0.008 to 0.044 γ (average 0.026 γ) of the amount of α -estradiol injected in each of five experiments. For 0.15 γ of α -estradiol standards, the values read from this line were within 0.002

TABLE I

Estradiol Inactivation by Various Preparations of Rat Liver

The inactivation shown was obtained with 50 mg. of liver and 0.3 γ of α -estradiol in each assay animal.

Preparation	No. of experiments	No. of assay animals	Average α -estradiol inactivated	Inactivation
			γ	per cent
A. Liver slices	11	39	0.26	87
B. " mince	13	49	0.09	30
C. " " + hot saline liver extract	5	16	0.25	83
D. " " + " aqueous liver extract	3	10	0.17	57
E. " " prepared in 0.1% nicotinamide	4	16	0.25	83
F. " " + 90 γ DPN added with α -estradiol	7	27	0.27	90
G. Liver mince + 90 γ DPN added 30 min. prior to α -estradiol	5	20	0.14	47
H. Liver mince, nicotinamide added to 0.1%, followed by 90 γ DPN, with α -estradiol 30 min. later	4	15	0.24	80
I. 90 γ DPN + α -estradiol (without liver)	5	20	0.01	3

to 0.038 γ (average 0.012 γ) of this amount of α -estradiol injected in each of five experiments. It was thus possible to compare the inactivation of α -estradiol in different experiments in terms of microgram equivalents.

Results

The average amounts of α -estradiol inactivated by various types of preparations of rat livers are summarized in Table I. Under the conditions employed, 50 mg. of liver slices (Preparation A) were able to inactivate an average of 0.26 γ of the 0.3 γ of α -estradiol added. Mince (Preparation B), prepared from these and other livers, was able to inactivate only 0.09 γ of the same amount of α -estradiol. For every liver tested, the efficiency of the slices was considerably greater than that of the mince.

These results suggested that a component of the inactivating system was unstable in the mince preparation employed and might have been destroyed by an enzyme released in the mince. Livers were sliced into hot physiological saline, boiled for 10 minutes, and, after cooling, the clear, yellow supernatant liquid was decanted from an amount of liver slices equivalent to the weight of liver to be used as mince. This extract was mixed with the estrogen prior to addition of the mince. The data in Table I show that this preparation (C) was approximately as efficient as the liver slices (Preparation A). The addition of a water extract of liver slices prepared in the same manner increased the inactivating ability of mince (Preparation D), but not to this degree.

Since DPN is known to be rapidly lost from minced tissues (22-24), experiments were carried out to test the possibility that DPN was the labile factor in this system. The destruction of DPN in tissue mince is inhibited in the presence of nicotinamide (23, 24). Accordingly, liver mince was prepared in a saline solution containing 0.1 per cent (0.0082 M) nicotinamide, and was tested for α -estradiol-inactivating ability. This preparation was also as effective as liver slices (Preparation E).

When 90 γ of DPN¹ were mixed with 0.3 γ of α -estradiol, prior to the addition of liver mince unprotected by nicotinamide (corresponding to Preparation B), this preparation (F) inactivated the α -estradiol as well as did liver slices, mince with added saline extract of liver, or mince prepared in nicotinamide solution. However, when unprotected mince was allowed to stand with the added DPN for 30 minutes at room temperature, prior to the addition of estrogen (Preparation G), only a small increment of inactivation over that observed for the same mince without added DPN (Preparation B) was obtained.

When 0.1 per cent nicotinamide solution was added to a similar mince before the DPN was added, this preparation (H) was able to inactivate α -estradiol more effectively than did Preparation G and as well as Preparations C, E, or F.

Incubation of 0.3 γ of α -estradiol with 90 γ of DPN in the absence of liver resulted in no significant degree of inactivation (Preparation I).

Fig. 1 illustrates the effect of varying concentrations of DPN on the α -estradiol-inactivating ability of liver mince in the presence or absence of added nicotinamide. The mince used in this experiment was prepared in saline and allowed to stand 30 minutes before its addition to the

¹ Obtained from the Schwarz Laboratories, Lot CO-4704, said to be of 60 per cent purity and free of triphosphopyridine nucleotide and flavin-adenine dinucleotide. This was found to contain 57 per cent DPN on the basis of its nicotinic acid content after hydrolysis. All amounts of DPN are expressed in terms of the content of active material.

incubation mixture in order to permit substantial destruction of DPN already present. The components were added to phosphate buffer in saline in the following order: estradiol, DPN, nicotinamide (when used), and liver mince. Inactivation of α -estradiol by mince (within the limits tested) appears to be related to the amount of DPN present. The addition of nicotinamide permits a more complete inactivation of estrogen in the presence of intermediate amounts of DPN.

The increased destruction of DPN with time (24) was confirmed for the conditions employed in this study. DPN content was assayed by the

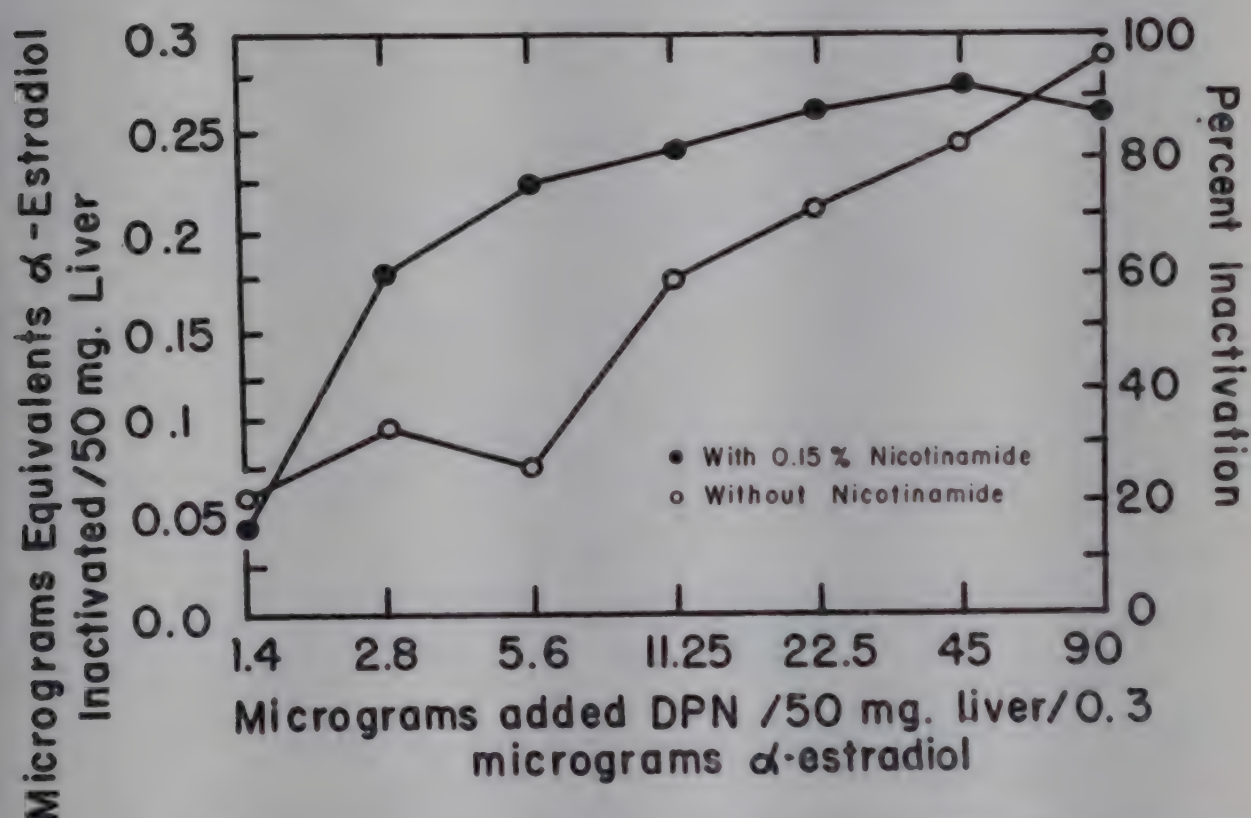


FIG. 1. Effect of nicotinamide and varying concentrations of DPN on inactivation of α -estradiol by liver mince.

method of Robinson *et al.* (25). In order to determine the original DPN level of the liver, a portion was removed and immediately blended in saline containing 2 per cent nicotinamide and 3 mg. of ceric sulfate per ml. to prevent DPN destruction (25). The remainder of the liver was blended in saline in the same manner. After this mince had been allowed to stand in the refrigerator for 10 minutes at 7°, a sample (B, Table II) was withdrawn and placed in a solution containing enough nicotinamide and ceric sulfate to give a final concentration of 2 per cent nicotinamide and 3 mg. of ceric sulfate per ml. To the remaining mince, 85 γ of DPN per 50 mg. of liver were added and blended for 5 seconds. After the mince had stood at room temperature for 10, 20, 30, and 45 minutes, samples were with-

drawn and placed in nicotinamide-ceric sulfate solution (Table II). These results show that DPN is rapidly destroyed by liver mince in saline under the same conditions used in the estrogen studies.

The effect of varying concentrations of nicotinamide upon the destruction of added DPN by liver mince containing α -estradiol is shown in Table III. Samples of liver mince (10 minutes after blending) were added to

TABLE II
Destruction of DPN by Liver Mince

Liver sample	Conditions	DPN per 50 mg. liver
		γ
A	Mince blended in nicotinamide-Ce(SO ₄) ₂ immediately after removal	84
B	Mince blended in saline and allowed to stand 10 min. at 7° 85 γ DPN per 50 mg. liver added to mince after removal of Sample B	16
C	Mince taken 10 min. after addition of DPN	23
D	" " 20 " " " " "	23
E	" " 30 " " " " "	17
F	" " 45 " " " " "	15

TABLE III
Effect of Nicotinamide Concentration on DPN Destruction by Liver Mince

Nicotinamide concentration	DPN found 10 min. after addition of 85 γ DPN per 50 mg. liver mince	DPN found 10 min. after addition of 21 γ DPN per 50 mg. liver mince
<i>per cent</i>	γ	γ
0	20	14
0.02	32	20
0.1	38	21
0.5	49	22
2.5	70	28

phosphate buffer in saline, followed by nicotinamide (when used), DPN, and α -estradiol. These mixtures were incubated for 10 minutes at 38° and a sample of each transferred into nicotinamide-ceric sulfate solution as before.

The results show that some destruction of DPN occurred with all levels of nicotinamide used, although the protection increased as the concentration of nicotinamide was raised. The 0.1 per cent nicotinamide solution used in the studies of α -estradiol inactivation afforded partial protection to the DPN (Table I). In an experiment similar to that shown in Fig. 1,

it was found that the estrogen-inactivating ability of liver mince fortified with a constant amount of DPN (90 γ per 50 mg. of liver) was increased with higher levels of nicotinamide. Minces containing 0.02, 0.1, 0.5, and 2.5 per cent of nicotinamide inactivated 80, 90, 100, and 100 per cent respectively, of the added α -estradiol. This agrees with the findings in Table III.

DISCUSSION

The ability of rat liver slices to convert α -estradiol to metabolites of lesser estrogenic activity is superior to that of rat liver mince. This difference in relative ability can be overcome by the addition of a boiled saline extract of rat liver or of purified DPN to the mince preparation. The protection of the DPN in rat liver and of the α -estradiol-inactivating ability of the liver afforded by preparing mince in nicotinamide solution also demonstrates the rôle of DPN in the metabolism of α -estradiol.

DPN is rapidly destroyed by a nucleosidase which is liberated from broken cell tissue preparations (22-24). The nucleosidase is most active at pH 7.2 (26) or 7.5 (24) which is similar to the pH of incubation (7.4) of liver preparations with the α -estradiol used in this study. The DPN content of liver mince which is blended in saline and allowed to stand for 10 minutes at 7° is about one-fifth of that found in the original liver. The addition of purified DPN to this preparation results in rapid inactivation of the added DPN. Nicotinamide inhibits the nucleosidase and protects the DPN. These data agree with previous findings on DPN nucleosidase (23, 24) and account for the loss of α -estradiol-inactivating ability of mince.

Procedures which are known to protect DPN in tissue preparations have been found, in the present experiments, to maintain the estrogen-inactivating ability of liver mince. The degree of inactivation of α -estradiol by liver mince has also been shown to be related to the level of DPN added to liver mince.

DPN has been shown to be necessary for the conversion of testosterone to 17-ketosteroids by liver mince (27). Estrone has been isolated as one of the metabolites resulting from the incubation of α -estradiol with rat liver slices (28) and has also been shown to be an end-product of *in vivo* metabolism of α -estradiol (29). It is possible that DPN is involved in the oxidation of α -estradiol to the ketosteroid, estrone.

SUMMARY

The addition of purified DPN or of a boiled saline extract of rat liver increases the α -estradiol-inactivating ability of rat liver mince to equal that of liver slices.

The ability of rat liver mince to inactivate α -estradiol is related to the amount of DPN added to the preparation.

When nicotinamide, which protects DPN, is used in the preparation of liver mince, the estrogen-inactivating ability of the liver is not lost.

The data suggest that DPN is involved in the metabolism of α -estradiol.

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BIBLIOGRAPHY

1. Zondek, B., *Skand. Arch. Physiol.*, **70**, 133 (1934).
2. Golden, J. B., and Sevringhaus, E. L., *Proc. Soc. Exp. Biol. and Med.*, **39**, 361 (1938).
3. Biskind, G. R., and Mark, J., *Bull. Johns Hopkins Hosp.*, **65**, 212 (1939).
4. Biskind, G. R., *Endocrinology*, **28**, 894 (1941).
5. Biskind, G. R., *Proc. Soc. Exp. Biol. and Med.*, **47**, 266 (1941).
6. Segaloff, A., *Proc. Soc. Exp. Biol. and Med.*, **60**, 108 (1945).
7. Segaloff, A., Nelson, W. O., *Proc. Soc. Exp. Biol. and Med.*, **48**, 33 (1941).
8. Segaloff, A., *Endocrinology*, **33**, 209 (1943).
9. Segaloff, A., *Endocrinology*, **34**, 335 (1944).
10. Talbot, N. B., *Endocrinology*, **25**, 601 (1939).
11. Pincus, G., and Martin, D. W., *Endocrinology*, **27**, 838 (1940).
12. Selye, H., *J. Pharmacol. and Exp. Therap.*, **71**, 236 (1941).
13. Segaloff, A., *Endocrinology*, **38**, 212 (1946).
14. Heller, C. G., *Endocrinology*, **26**, 619 (1940).
15. Twombly, G. H., and Taylor, H. C., Jr., *Cancer Res.*, **2**, 811 (1942).
16. Singher, H. O., Kensler, C. J., Taylor, H. C., Jr., Rhoads, C. P., and Unna, K., *J. Biol. Chem.*, **154**, 79 (1944).
17. Engel, P., and Rosenberg, E., *Endocrinology*, **37**, 44 (1945).
18. Levy, H., *Arch. Biochem.*, **14**, 325 (1947).
19. Coppedge, R. L., Segaloff, A., Sarett, H. P., and Altschul, A. M., *J. Biol. Chem.*, **173**, 431 (1948).
20. DeMeio, R. H., Rakoff, A. E., Cantarow, A., and Paschkis, K. E., *Endocrinology*, **43**, 97 (1948).
21. Lauson, H. D., Heller, C. G., Golden, J. B., and Sevringhaus, E. L., *Endocrinology*, **24**, 35 (1939).
22. Myrbäck, K., and Nilsen, R., *Z. physiol. Chem.*, **165**, 140 (1927).
23. Mann, P. J. G., and Quastel, J. H., *Biochem. J.*, **35**, 502 (1941).
24. Handler, P., and Klein, J. R., *J. Biol. Chem.*, **143**, 49 (1942).
25. Robinson, J., Levitas, N., Rosen, F., and Perlzweig, W. A., *J. Biol. Chem.*, **170**, 653 (1947).
26. Spaulding, M. E., and Graham, W. D., *J. Biol. Chem.*, **170**, 711 (1947).
27. Sweat, M. L., and Samuels, L. T., *J. Biol. Chem.*, **175**, 1 (1948).
28. Pearlman, W. H., and DeMeio, R. H., *Federation Proc.*, **8**, 238 (1949).
29. Jailer, J. W., *J. Clin. Endocrinol.*, **9**, 57 (1949).

ON THE UNLIKELYHOOD OF SPECIFIC LONG RANGE FORCES IN IMMUNOLOGIC AND ENZYMATIC REACTIONS*

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PLATES 1 AND 2

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In the past few years, a great many experiments have been performed by Rothen (24, 25) which he has interpreted as suggesting that antigen and antibody, and enzyme and substrate proteins, can react specifically, although separated by distances of hundreds of angstroms. This hypothesis seems to conflict with evidence accumulated from many other investigations indicating that short range interactions, involving distances usually associated with electrostatic and van der Waals' forces, and hydrogen bonds, of the order of 5 Å, operate in these reactions. We have therefore engaged in a critical analysis of the experimental techniques used by Rothen to determine whether some interpretation other than that invoking the existence of specific long range forces could be found to explain his experimental results.

Before we proceed with the details of this investigation, it might be worth while to summarize briefly the evidence at hand that short range forces are primarily responsible for the specificity of immunologic and enzymatic reactions. The work of Landsteiner (15), Haurowitz (9), Pauling and his collaborators (21), and others, using as antigens proteins to which known chemical groups (haptens) had been chemically conjugated, has demonstrated the very high order of this specificity in antigen-antibody reactions. Antisera were prepared which contained antibodies directed specifically against a particular haptenic group, and these antisera were allowed to react with a large number of antigens containing somewhat different haptens. By a detailed systematic study of these reactions it was demonstrated that slight structural changes in the hapten (slight, that is, from any long range point of view), such as the substitution of a carboxyl group for an arsonic acid group on a benzene ring or the replacement of an L-tartranilic acid hapten for the D isomer, could have

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profound effects on the extent of the cross-reactions observed. Moreover, Landsteiner discovered that the low molecular weight hapten itself, not associated with a protein, could specifically inhibit the precipitation of the antibody and the hapten-conjugated antigen.

After a more quantitative investigation of these phenomena, Pauling, Pressman, and Campbell (20) proposed a mechanism for the antigen-antibody reaction, more explicit than earlier ones, requiring complementary configurations for the antigen and antibody, so that, over a considerable portion of both molecules, short range van der Waals', electrostatic, hydrogen bond, and similar forces could cooperate to form a bond that was cumulatively sufficiently strong. The phenomenon of hapten inhibition of antigen-antibody precipitation could be ascribed to the competition between hapten and hapten-conjugated antigen for the complementary sites on the antibody. This hypothesis seems to be in accord with most of the experimental results in immunochemistry, as well as with the existing knowledge about the nature of the forces involved in systems of small molecules.

In the case of enzymatic reactions direct evidence has been obtained in certain cases for the existence of short range mechanisms. In recent years it has been possible to demonstrate spectroscopically the existence of certain activated complexes between enzyme and substrate, as suggested long ago by Michaelis and Menten (18). These authors proposed that enzyme and substrate form an unstable compound, the activated complex, which subsequently rearranges to yield the original enzyme plus the reaction products. Keilin and Mann (14) studied the complex formed between peroxidase and hydrogen peroxide, and Chance (5) that between catalase and hydrogen peroxide, by changes in the absorption spectra produced on the formation of the complex. A great amount of material concerning the high order of specificity of various peptidases (1) and their metal ion requirements (27) can also be very well explained by this mechanism, involving the formation of short range chemical bonds between enzyme and substrate molecules.

It would appear, after careful consideration of the facts that have been mentioned only briefly here, that the hypothesis of long range forces requires either of two improbable alternatives, (a) either that several or a whole range of mechanisms involving a spectrum of short and long range forces is operative in both immunologic and enzymatic reactions or (b) that the principles of structural chemistry acquired through the study of systems of small molecules, which have been used to interpret the evidence cited above, are totally inadequate to explain the reactions of large molecules.

The experiments of Rothen constitute the only evidence that has been

advanced to demonstrate directly that specific long range forces exist. If these experiments can be interpreted satisfactorily by other means, then, in consideration of the improbable alternatives afforded us by the concept of long range forces, we would not be justified in presuming that such interactions exist.

We have obtained experimental evidence that strongly suggests that the results of Rothen can be interpreted by a much simpler mechanism that does not require the existence of long range forces. This evidence and its interpretation make up the major part of this paper. In order to discuss these, we must first describe the experimental techniques and results obtained by Rothen.

Rothen's Technique and Results

The experiments from which the hypothesis of long range forces evolved were concerned with the preparation and accurate measurement of very thin films of antigens, antibodies, and various inert materials. (Henceforth we shall restrict most of our discussion to the immunologic reaction, unless otherwise specified.) On a suitable substrate, such as a highly polished stainless steel slide, an optical gage of barium stearate was deposited by the Blodgett-Langmuir technique (2). This gage consisted of two monolayers of barium stearate on the top half of the slide and four monolayers on the bottom half.¹ The optical gage permitted the use of the half shadow principle with an instrument, developed by Rothen (22), called the "ellipsometer," which is capable of accurate measurements of film thicknesses. The slide with the optical gage was then "conditioned" by contact with a uranyl acetate-veronal buffer solution, which made it possible subsequently to pick up, by the Blodgett-Langmuir technique, more than two monolayers of bovine serum albumin antigen. After the deposition of the antigen layers, the surface was covered with a thin film of inert material (the barrier or screen) such as Formvar, barium stearate, or octadecylamine. A solution of antiserum was then placed on this surface, and allowed to react for several minutes; the slide was then washed in a reproducible manner and dried, and the increment in total film thickness was measured.

By means of this technique, or modifications of it, Rothen obtained the following results. First, let us consider those experiments in which antibody was adsorbed directly on the antigen without intervening barrier films being present. Although in the case of the egg albumin-anti-egg albumin rabbit serum system the amount of antibody specifically adsorbed directly on the antigen was independent of the number of monolayers of antigen on the slide, in the bovine serum albumin-antiserum system the

¹ In our experiments we used three and five monolayers for the optical gage.

amount of antibody adsorbed increased linearly with the number of monolayers of antigen up to eight layers. It was argued that thickness measurements demonstrated that the antigen films consisted of completely unfolded molecules, since the thickness per monolayer was found consistently between 8 and 9 Å. Under these circumstances the topmost layer of antigen should have protected underlying layers from the action of antibody if only short range forces were involved, and the amount of antibody adsorbed should have been independent of the number of layers of antigen on the slide. This was not the case in the bovine serum albumin-antiserum system. Other experiments dealt with the pneumococcus type III polysaccharide and rabbit antisera. In certain cases as much as 700 Å of antibody were adsorbed on only 5 Å of the polysaccharide, and from these experiments it was argued that long range forces were specifically immobilizing antibody which could not have come into direct contact with antigen.

In his experiments with inert barrier films interposed between antigen and antibody, Rothen used Formvar, barium stearate, and octadecylamine screens, the latter two applied by the Blodgett-Langmuir technique. He found that the amount of antibody adsorbed on top of the barrier covering the antigen fell off approximately linearly with barrier thickness, and was independent of the nature of these barriers within the experimental errors involved. In subsequent experiments with enzymatic reactions (25), however, he found that thin films of gold, evaporated from a hot filament in a high vacuum, were effective in inhibiting the reaction.

Considering the possibility that holes in the barrier films were responsible for the effects observed, Rothen attempted to show that they did not exist, or in any event were not important. We shall defer presenting these arguments until the section under "Discussion," and proceed to the experimental part of our investigation.

Experimental Results and Interpretation

We have obtained evidence that strongly suggests that holes in the barrier films are indeed responsible for most of the effects observed. Three different approaches have been utilized: (a) an investigation of certain barrier films other than those used by Rothen, (b) an electron microscope study of the various surfaces involved in these experiments, and (c) experiments with hapten-conjugated protein antigens and antibodies specifically directed against the hapten group.

Barrier Films—In order to carry on this investigation as effectively as possible we attempted to duplicate Rothen's technique in complete detail. We built an ellipsometer² of comparable sensitivity for film thickness

²Made to our specifications by the Instrument Development and Manufacturing Company, 3018 East Foothill Boulevard, Pasadena, California.

measurements and the necessary troughs and auxiliary equipment. In only two minor regards did our technique differ from Rothen's: First, instead of using highly polished stainless steel slides as the film substrates, we used carefully cleaned microscope glass slides coated with a film of aluminum approximately 1000 Å thick. The aluminum was evaporated on both sides of the slides in a high vacuum mirror-coating assembly. We found these slides considerably easier to prepare than the stainless steel slides, and moreover we were able to discard them after one use. In some of his later experiments Rothen had reported (25) that chromium-coated or gold-coated glass slides gave the same results as those obtained with stainless steel slides. Secondly, in the deposition of the protein monolayers on to the conditioned barium stearate surface, Rothen used a movable bar and a Wilhelmy balance on the trough to maintain the film pressure at 8 dynes cm^{-1} . Instead, we used a paraffined mercerized cotton thread as the barrier to contain the protein film, and purified triorthocresyl phosphate³ as the piston oil, in the usual manner of depositing monolayer films (2). Proper care was taken to eliminate the possibility of contaminating the protein film with the oil. Langmuir and Schaefer (17) have recommended the use of triorthocresyl phosphate as a piston oil, and report its spreading pressure to be 9.5 dynes cm^{-1} . The difference between 8 and 9.5 dynes cm^{-1} is not significant for our purposes. However, we found that, while we were able to pick up a double layer on the first down and up trips of the slide through the compressed protein monolayer, further layers were picked up only after allowing the slide to dry and then only on the up trips. This is somewhat different from Rothen's findings, since he was able to pick up a layer on each down trip and up trip. This slight discrepancy, however, presumably has little effect on the results obtained.

Our experiments with different barrier films are recorded in Table I and Text-fig. 1.⁴ To avoid too much detail in Text-fig. 1 we have included experimental points only in the cases of Curves A and G. These experiments were all performed with four layers of bovine serum albumin (Armour) as the underlying antigen film. The pooled rabbit antiserum, provided through the kindness of Professor Dan H. Campbell, contained about 5 mg. of antibody per ml., as determined by quantitative precipitin

³Technical triorthocresyl phosphate was purified by extensive washing with dilute NaOH, and then with water, and was then dried over CaCl_2 . The oil distilled at about 200° at 350 μ pressure.

⁴Formvar is a polyvinyl alcohol partially converted to the acetal by treatment with formaldehyde, and is sold by the Shawinigan Products Corporation, New York. VYN-W is a vinyl chloride-vinyl acetate copolymer manufactured by the Bakelite Corporation, New York. The ethyl cellulose and the cellulose acetate, designated N-22 and LL-1 respectively, were obtained from the Hercules Powder Company, Wilmington, Delaware. Parlodion is a partially esterified nitrocellulose supplied by the Mallinckrodt Chemical Works, St. Louis.

TABLE I
Experiments with Different Barrier Films

Nature of barrier	Barrier* thickness, A	Antibody† adsorbed, A	Nature of barrier	Barrier thickness, A	Antibody adsorbed, A
No barrier	0	81 ± 4‡	Ethyl cellulose in	28	55
Formvar in ethylene chloride (Curve A)	17	67	acetone (Curve D)	33	40
	39	36		35	40
	43	61		53	35
	54	56		54	49
	84	32		56	18
	92	40		58	35
	94	24		61	56
	94	48		64	44
	102	18		69	41
	104	24		80	37
	125	26		88	13
	133	34		90	30
Barium stearate (Curve B)	48 ± 4§	56 ± 10§	Cellulose acetate in acetone (Curve E)	101	34
	92 ± 4§	33 ± 7§		111	25
VYN-W in methyl- ethyl ketone (Curve C)	8	65		111	4
	46	65		10	65
	46	49		17	65
	69	53		25	43
	71	65		45	50
	78	32		48	17
	83	48		48	54
Parlodion in methyl- ethyl ketone (Curve F)				49	20
				58	26
				62	26
				75	22
				77	13
				78	0
				81	0
				85	20
	13	20	Ethyl cellulose in ethylene chloride (Curve G)	13	20
	23	66		14	24
	28	59		15	48
	29	36		15	27
	30	30		20	28
	31	32		22	49
	32	30		22	31
	41	40		30	5
	42	19		41	22
	49	30		44	9
	55	29		50	0
	57	13		58	0
	64	13		72	0
	69	10		73	5

TABLE I—*Concluded*

Nature of barrier	Barrier* thickness, A	Antibody† adsorbed, A	Nature of barrier	Barrier thickness, A	Antibody adsorbed, A
Parlodion in methyl- ethyl ketone (Curve F)—con- tinued	71	0	Ethyl cellulose in ethylene chloride (Curve G)—con- tinued	76	3
	80	10		79	0
	88	32		111	0
	122	9		115	0
	136	0		120	0

* Acetone, methylethyl ketone, and ethylene chloride, when applied to the slides on which the antigen was deposited on the conditioned optical gage, caused an apparent decrease of about 10 A in the measured film thickness, presumably due to the solubilization of some of the stearic acid in the optical gage by the organic solvents. Higher boiling solvents, like amyl acetate, completely obliterated the line of demarcation between the top and the bottom half of the optical gage. The measured barrier thicknesses given, except in the case of barium stearate, are therefore somewhat smaller than the true values.

† Control experiments in which normal rabbit serum was used in place of anti-serum, under otherwise identical conditions, resulted in a small decrease, about 5 to 10 A, in the total film thickness. No correction was made for this effect, however.

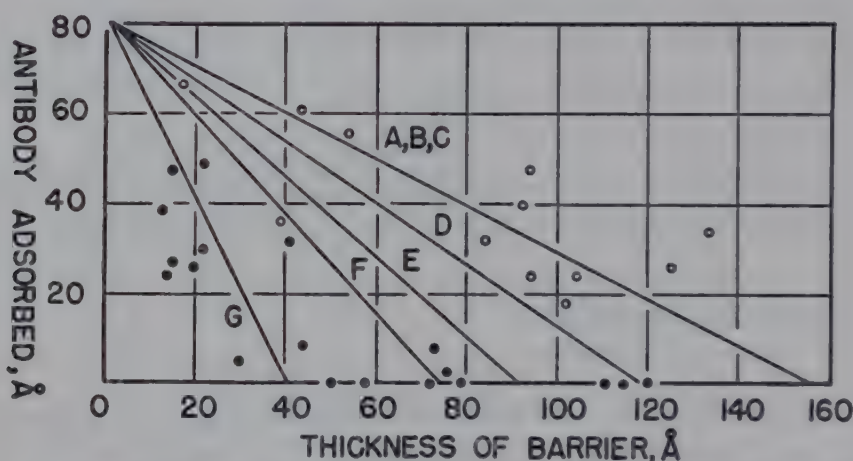
‡ Average of twenty experiments.

§ Average of four experiments.

tests. It was kept sterile, and aliquots were diluted 1:6 with a phosphate-saline buffer just before use. This buffer is the same as that described by Rothen. The precision of the data obtained in the experiments with polymer barrier films is not high. This lack of precision appears to be characteristic of the technique; yet it is not due to any irregular behavior of the antigen layers or the antiserum, since the results of the experiments in which antibody was adsorbed directly on four layers of bovine serum albumin were considerably more precise. In twenty experiments performed on independent batches of slides, an average of 81 A of antibody was adsorbed on the antigen with an average deviation from the mean of ± 4 A. In order to ascertain that differences in antibody adsorbed on different barrier films were real and not due to possible batch to batch variations, each set of slides was usually divided into two subsets after the deposition of the antigen layers, and then one subset was coated with barrier films of one polymer, the other subset with films of another, and antibody adsorption studied on both types of barriers at the same time. Often one of the experiments in which antibody was adsorbed directly on the antigen was also performed on one of the slides in a set. In view of the experimental scatter, the straight lines we have drawn to characterize the data are not to be taken too seriously; nevertheless, certain features of these results are significant and worth noting.

The curve we obtained with Formvar screens (Curve A) is roughly parallel to the one obtained by Rothen under similar conditions; the lack of duplication of these curves is accounted for by the use of different antisera in the two studies. Moreover, we find that, within the experimental error, the results obtained with Formvar and barium stearate screens coincide. These data confirm those of Rothen, and indicate that our experimental technique effectively duplicates his.

A family of curves was obtained, depending upon the particular cellulose derivative used as the inert barrier. Despite the experimental scatter, there is no question but that less antibody was adsorbed on ethyl cellulose films cast from ethylene chloride than was adsorbed on parlodion and cellulose acetate films of the same thickness; and in turn less antibody was



TEXT-FIG. 1. Screening action of various materials deposited on four layers of bovine serum albumin. Curve A, Formvar in ethylene chloride; Curve B, barium stearate; Curve C, VYN-W in butanone; Curve D, ethyl cellulose in acetone; Curve E, cellulose acetate in acetone; Curve F, parlodion in butanone; and Curve G, ethyl cellulose in ethylene chloride. ○, the data through which Curve A is drawn; ●, the data for Curve G.

adsorbed on these films than on those of Formvar. On the average, films only 40 Å thick of ethyl cellulose cast from ethylene chloride sufficed to inhibit the adsorption of antibody.

It was found that the same polymer, ethyl cellulose, in two different solvents, ethylene chloride (Curve G) and acetone (Curve D), gave two entirely different curves.

Let us consider the meaning of these results. The cellulose derivatives used in this study differ from one another chemically only in the replacement of certain groups for similar ones along the cellulose chain. The chemical fabric of these cellulose derivatives is not very different from that of Formvar; in fact, these derivatives are chemically much more like Formvar than Formvar is like barium stearate, and yet the latter two exhibit the same behavior when used as inert barriers. It would indeed

be astonishing if the postulated long range forces were susceptible to such large fluctuations upon changing the nature of the dielectric medium so slightly. It is more probable to conclude that the *physical states* of these films, which might very well depend on such slight chemical changes in the cellulose chains, are considerably different. Other evidence supporting this view is presented in the section on electron microscopy.

It is also difficult to understand, from the point of view of long range forces, how films of the same dielectric material, ethyl cellulose, can behave in two such radically different ways when cast from two different solvents. The conclusion would seem unavoidable that the physical state of the barrier film is of fundamental importance, and that films cast from ethylene chloride are better films than those prepared from acetone solution. It is known from the osmotic pressure and viscosity studies of Spurlin, Martin, and Tennant (28) that ethyl cellulose is appreciably associated, that is, not molecularly dispersed, in acetone solution, while it is probably not associated in ethylene chloride. The presence of aggregates presumably prevents the film cast from acetone solution from having as uniform a structure as the one cast from ethylene chloride. And yet Curve D is not very different from that obtained with Formvar screens (Curve A); nor do the surfaces of such ethyl cellulose films appear to contain holes or be otherwise unusual when examined in the electron microscope.

Two other explanations might be advanced to account for this family of curves. First, partial denaturation of the antigen might somehow have occurred in these experiments differing in extent from one barrier film to the next. This is not likely, however, since the same solvent, ethylene chloride, was used in the experiments of Curves A and G; nor is there any reason to suspect that the polymers themselves have any specific deleterious effects on the antigen or antibody, particularly since the same polymer, ethyl cellulose, exhibited the behaviors characterized by the two curves, D and G. Secondly, in using the ellipsometer, we assumed all films to have the same refractive index in order to measure their thicknesses, and this assumption might at first sight seem objectionable. However, Rothen has shown (24) that variations in the refractive index such as exist among the various polymers used in this study have only a small effect on the results of the thickness measurements.

We have also examined evaporated films of silica as inert barriers. Electron microscope studies have revealed the relatively uniform structure of such films (10); moreover from the similarity between these amorphous SiO_2 films and glass, which is known to have a surface smoother than that of any polymer film (29), it may be inferred, although it has not yet been conclusively demonstrated, that silica films are more uniform in structure than those of Formvar or parlodion. The silica (b.p. 2200°) was evapo-

rated from a hot tungsten basket filament at a distance of about 15 cm. from the antigen-covered slide, at a pressure between 10^{-3} and 10^{-4} mm. of Hg. Films of 18 Å or more of silica,⁵ deposited directly on four layers of bovine serum albumin, completely inhibited the subsequent adsorption of antibody. In order to determine whether this was an artifact due to inactivation of the antigen, the following experiments were performed. Under identical conditions, CaCl_2 (b.p. $>1600^\circ$) was evaporated directly on the antigen layers, and was subsequently dissolved by the antibody solution placed on top of it. The same amount of antibody, about 80 Å, was found to be adsorbed in these experiments as in the usual ones in which antibody solution was placed directly on the antigen. This indicates that neither the high vacuum nor the radiation energy from the tungsten filament played any rôle in the inhibition of antibody adsorption by silica barriers. Experiments were also performed in which the antigen layers were first covered with Formvar films about 40 Å thick, and then 18 Å or more of silica was deposited on the Formvar. Such combined films also completely inhibited the reaction,⁶ although 60 Å of Formvar alone permitted 50 Å of antibody to be adsorbed (Text-fig. 1). The same results were obtained when six, instead of four, layers of bovine serum albumin antigen were used. These experiments demonstrate that the inhibition of the reaction by silica films is not due to some kind of chemical inactivation of the antigen by the silica.

Attempts to use other films evaporated onto the antigen surfaces ran into difficulties. Intervening films of TiO_2 25 Å thick permitted only 10 Å of antibody to be adsorbed on four layers of bovine serum albumin. TiO_2 , however, appears to liberate oxygen when heated to its evaporation temperature; the pressure inside the evaporation assembly rose sharply upon heating the filament containing the dry TiO_2 . No such change of pressure occurred on evaporating SiO_2 . MgF_2 was found to be too soluble in water. Barriers of AgCl about 50 Å thick permitted 25 Å of antibody to be adsorbed on four layers of bovine serum albumin. On examination in the electron microscope, however, the film of AgCl was observed to be crystalline, unlike that of silica, and accordingly it would not be expected to be as effective a screen. On the other hand, it is considerably more effective than Formvar.

Electron Microscope Studies—The barrier films used in this investigation were too thin to be examined by direct transmission in the electron micro-

⁵ Silica has a similar refractive index, around 1.46, to the protein and polymer films.

⁶ Control experiments with normal sera indicated that, while either Formvar or silica films on the antigen did not adsorb measurable amounts of protein, silica films on Formvar films on the antigen adsorbed about 12 Å of protein nonspecifically under similar conditions.

scope. Apart from their fragility in thicknesses of the order of 100 Å, the degree of contrast between the films and any small holes that might exist within them would be too low to be observed with any certainty. We therefore resorted to examining the various films involved in this study by making replicas of their surfaces by well known shadow-casting techniques (29), and observing these replicas in the microscope.

We have reproduced several of these micrographs in Figs. 1 and 2. Except for the first specimen, all of the surfaces studied were directly replicated with chromium at a shadowing angle whose tangent was about one-fifth and were subsequently backed up with a polymer film to lend the requisite strength to the replica, and the metal and polymer films were then stripped from the underlying surfaces with Scotch tape (29). In examining the surface of the aluminum film on glass, the mirror substrate used in all our experiments, a parlodion replica of the surface was cast from amyl acetate solution and was stripped with Scotch tape, and this replica was then shadowed with chromium.

In the course of these studies we found it impossible to strip parlodion-backed chromium replicas from a considerable number of surfaces. Upon substituting ethyl cellulose films cast from ethylene chloride solution for the parlodion, we were able to strip the metal from nearly all the substrates we tried. For example, one to six monolayers of bovine serum albumin on a conditioned optical gage of barium stearate on the aluminum-glass substrates completely retained parlodion-backed chromium films, but easily permitted ethyl cellulose-backed replicas to be stripped. Certain surfaces, such as those of Formvar on top of such antigen layers, permitted both types of polymer-backed replicas to be stripped, but in no case was it possible to remove parlodion-backed chromium replicas from surfaces that retained those backed with ethyl cellulose films cast from ethylene chloride. This improvement in the replica technique has been mentioned elsewhere (26). It is interesting that ethyl cellulose films are more effective in this stripping procedure than are parlodion films, which are in turn more effective than Formvar films. This adhesiveness to the chromium films is in the same order as the capacity of these films to inhibit the adsorption of antibody on underlying antigen layers. It would seem likely that this correlation is due to the physical structure of these films; the more closely knit the structure, the stronger the short range van der Waals' attractions between the polymer and the metal, and the greater the adhesiveness; and the more closely knit the structure, the fewer holes or loci capable of forming holes to permit the antigen and antibody to come into direct contact.

A direct chromium replica of the surface of four monolayers of bovine serum albumin on five layers of barium stearate on the aluminum-glass

substrate is reproduced in Fig. 1, *b*. Separate micrographs have shown the barium stearate layers to have little structure compared to that of the bovine serum albumin layers. The structure of the protein film is characteristic, and different from that of the underlying substrate. A micrograph of a similar bovine serum albumin surface was recently published by Karush and Siegel (13). However, the deposition of bovine serum albumin layers in their experiments was not accompanied by measurement of the optical thickness of the layers. They were therefore unable to prove that their films were the same as those prepared by Rothen. In our case, however, the films were measured with the ellipsometer, and, despite the fact that the surface was quite rough, each layer produced an increment in the ellipsometer reading corresponding to 9 Å.

We have therefore demonstrated that measuring 9 Å per layer of protein deposited is no proof that the layers on the slide are uniformly spread out. On the contrary, we have shown in these experiments that the ellipsometer is unable to distinguish between a smooth film and an irregular one of about the same average thickness, when the irregularities are much smaller than the wave-length of light. This situation can be understood theoretically as well, as has been demonstrated by Weigle.⁷ Let us suppose that the film on the metal surface consists of patches of larger thickness than that of the hypothetical uniform film, but that the patch dimensions and the average distance of separation from each other are smaller than the wave-length of light. From consideration of the Lorentz-Lorenz equation, it can be shown that the effective refractive index, n' , of the patchy film is reduced and is given by $(n'^2 - 1)/(n'^2 + 2) = f((n_1^2 - 1)/(n_1^2 + 2))$, where f is the fraction of the metal surface covered by the film and n_1 the refractive index of the film material. Rothen has stated (24) that, under the conditions of operation of the ellipsometer, the ellipse of polarization is mainly affected by changes in the phase difference between the two components vibrating in and perpendicular to the plane of incidence (22). This phase difference is given by $\Delta = -A(1 - (1/n^2))l$, where A is a constant, n the refractive index, and l the thickness of the uniform film. Upon substituting a patchy film for a uniform one, while the thickness of the patches is now greater than that of the uniform film, the effective refractive index is smaller. The net result may be, therefore, as Weigle has shown, that very little change is produced in the ellipse of polarization because of a balancing of these two effects, and hence it would be difficult with this technique to distinguish between smooth and patchy films.

The significance of this proof that the antigen layers are not smooth and uniform structures is that it explains the experiments in which more antibody was adsorbed, the greater the number of layers of bovine serum

⁷ Weigle, J. J., personal communication.

albumin. For, since the layers did not completely cover underlying ones, considerably more antigen than just the topmost layer was exposed to the action of antibody. This explanation was suggested by Karush and Siegel (13), and earlier by Kabat (12) and Pauling (19).

In Fig. 1, *c* and *d*, we have reproduced replicas of the surfaces of antibody adsorbed directly on five layers of bovine serum albumin, and of a Formvar film on the antigen, respectively, while in Fig. 2 there is shown a replica of the surface of antibody adsorbed on Formvar films on top of the antigen. It is to be noted that, while the first two are relatively free of any large irregularities, the last one shows that antibody is aggregated when adsorbed on bovine serum albumin films that have been covered with Formvar. These aggregates are not present in the original antisera, nor are they artifacts produced by the Formvar films. (The specimen of Fig. 1, *d* was washed with phosphate-saline buffer and water, and dried in the same manner as those of Figs. 1, *c* and 2.) The particular areas of the replicas that have been pictured are representative of the entire specimens, and all of these pictures have been reproduced several times under a variety of conditions.

If long range forces were operating between antigen and antibody, one would expect the antibody to be spread uniformly over the Formvar film above the antigen layers. Instead, the presence of aggregates strongly suggests the formation of antigen-antibody precipitates about local sites, presumably holes, in the Formvar films. With barium stearate screens (Fig. 1, *e*) it is not so easy to discern discrete antigen-antibody aggregates, possibly because the holes present or produced in these films are smaller and closer together (see "Discussion" below). Electron micrographs of antibody deposited on parlodion and cellulose acetate barriers on top of the antigen showed even smaller irregularities.

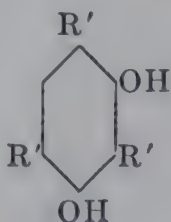
Conjugated Protein Antigen-Antisera Systems—At the beginning of this paper we outlined the immunochemical evidence, largely based on studies of hapten-conjugated proteins and antisera directed against the hapten groups, that indicates that short range forces operate in antigen-antibody reactions. The hapten groups are well known chemical structures, and there is no doubt concerning the fact that antibody activity is directed specifically against them. In the case of bovine serum albumin and similar molecules, however, no one has as yet been able to locate or define any antigenic foci on the molecules. This is not surprising, since so little is known about the detailed structure of proteins in general. Lacking this direct evidence for the existence of short range forces in these systems, however, we must at least recognize the possibility that Rothen's results with these systems and the great body of work involving conjugated proteins could be reconciled by postulating that a whole set of mechanisms

might be involved, ranging from a long range one in systems of the type of bovine serum albumin and its antibodies to a short range interaction in conjugated protein systems. If this were true, thin Formvar films should prevent antibody directed against the hapten from being adsorbed on films of hapten-conjugated protein antigens, if the Formvar films were actually preventing the antigen and antibody molecules from coming into direct contact.

In order to investigate this problem we performed some experiments with *p*-azophenylarsonic acid-conjugated proteins (henceforth referred to as R-azo proteins) and antibodies specifically directed against this hapten. Rabbit antisera were produced against R-azo bovine globulin, and R-azo gelatin and R-azo human albumin were used as test antigens.⁸ The conjugated antigens were prepared in the usual manner by slowly adding cold diazotized arsanilic acid to a cold dilute solution of the protein, keeping the pH about 9 by adding the necessary amount of dilute NaOH. The resultant mixture was placed in the refrigerator overnight, and was then exhaustively dialyzed against cold distilled water until no further color was observed in the solution outside the dialysis bags. On analysis the R-azo gelatin and R-azo human albumin yielded about 0.8 per cent As.

These conjugated proteins would not spread on distilled water, apparently because the introduction of arsonic acid groups had affected the solubility properties of the original proteins. They spread on buffers adjusted to pH ~ 4 , but it was felt that there was no particular advantage in picking up films spread on buffered solutions over adsorbing these proteins directly on the slides from solution. A drop of a 2.4 per cent solution of R-azo gelatin, or a 0.9 per cent solution of R-azo human albumin in water, was accordingly smeared on a uranyl acetate-condi-

⁸ The proteins and the anti-R-azo bovine globulin serum were obtained from Professor Campbell. The antiserum on analysis yielded about 1.5 mg. of antibody per ml. in precipitin tests with the dye antigen,

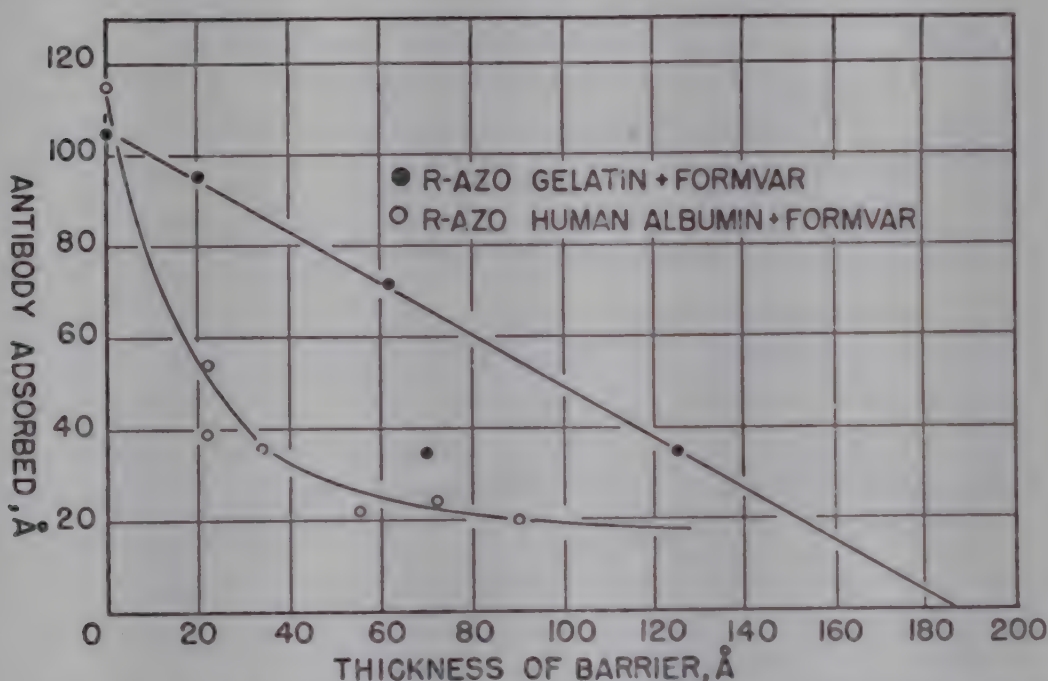


where $R' = -N=N-$  $-N=N-$  AsO_3H_2 (4). The bovine globulin,

obtained from Armour, was designated as Fractions II and III, the human albumin as Fraction V; and the gelatin was a specially prepared sample obtained from the Knox Gelatin Company, designated C-1, and characterized in blood substitute studies (3).

tioned optical gage of barium stearate and allowed to remain for a few minutes in a moist atmosphere. The unadsorbed residue was then washed off with distilled water. Under these conditions about 18 Å of the R-azo gelatin and 45 Å of the R-azo human albumin were fairly reproducibly adsorbed. Undiluted anti-R-azo bovine globulin serum was employed in the adsorption experiments, and the slides were washed and dried in the manner described previously.

The results of antibody adsorption experiments are given in Text-fig. 2. Each of the points represents an average of about four independent determinations. Antibody specifically directed against the *p*-azophenyl-arsonic acid group was adsorbed on the hapten-conjugated protein anti-



TEXT-Fig. 2. Anti-R-body adsorption on films of R-azo proteins coated with Formvar barrier films.

gens, despite intervening films of Formvar, in a manner similar to that found for the bovine serum albumin-antiserum system. Control experiments demonstrated that none of the antibody in the antiserum used in these experiments was adsorbed on the original human albumin and gelatin proteins when they were treated in the same manner, and no measurable amounts of normal serum proteins were adsorbed on the Formvar-coated films of the R-azo proteins. The shape of the R-azo human albumin curve is unusual, but there is no doubt that antibody specifically directed against the R group was adsorbed on the Formvar-covered antigen throughout the range of barrier thickness indicated.

These experiments provide us with the following alternatives. If holes in the barrier films are *not* responsible for the effects observed, then the long range force hypothesis must now be able to explain *all* the results

concerning the nature of the specificity of the conjugated protein-antibody system, as well as the phenomenon of hapten inhibition of antigen-antibody precipitation. On the other hand, if one is convinced by the available evidence that short range forces control the specificity of the reactions of these conjugated protein systems, then these experiments prove that antigen and antibody must be coming into direct contact through holes in the Formvar films. We have eliminated the possibility of a range of mechanisms in the immunochemical reaction such as we discussed at the beginning of this section.

DISCUSSION

Before discussing the interpretation of our experimental results in greater detail, let us consider the arguments presented by Rothen to show that holes in the barrier films are not responsible for the effects observed.

The results of Germer (8) on the study of films of barium stearate and stearic acid by electron diffraction are quoted to imply that these films are very uniform. However, the fact that these diffraction diagrams are usually characterized by diffuse bands, and by the absence of sharp spots, definitely indicates the lack of uniform, crystalline order in these films. The presence or absence of a small proportion of holes in these layers could not be ascertained by this method. More recent investigations by Williams and Epstein (7)⁹ with electron microscope and electron diffraction techniques indicate that monolayers of barium stearate consist of closely packed, pyramidal micelles about 100 Å in diameter, rather than of the more or less continuous structure ascribed to them by Germer. This would make for a film of considerably less cohesive strength, and with an increased probability for hole formation, than would be expected for a uniform film.

The suggestion made by Iball (11) might be introduced here. He proposed in effect that, even if a film of barium stearate monolayers deposited on antigen were free of many holes initially, then, under the stresses produced by the growth of antigen-antibody precipitates in relatively few places, considerably more holes or cracks could develop in the fragile film to permit more antigen and antibody to come into contact. This suggestion might be extended to apply to barrier films of Formvar and other polymers as well.

The fact that the same antibody adsorption curves were obtained with screens of barium stearate, octadecylamine, and Formvar is regarded by Rothen as more than coincidental. Our results with cellulose derivatives, however, show that the extent of antibody adsorption is definitely a function of the nature of the screen. Our electron micrographs imply

⁹ Williams, R. C., and Epstein, H., personal communication.

that the holes in the Formvar screens are larger but less frequent than those in the barium stearate layers, and that it is merely a coincidence that both screens permit the same amount of antibody to be adsorbed within the large experimental errors involved. It is not at all difficult to appreciate that a polymer film about 100 Å thick should, more or less independently of the nature of the surface on which it is cast, exhibit a certain number of holes large enough to permit antigen or antibody molecules to pass through. These films consist of several layers only of randomly coiled molecules with very little order among them. Holes in barium stearate films, considering the structure of these monolayers proposed by Williams and Epstein, might be expected to be more closely spaced and more uniformly distributed.

The argument is advanced that, if there are holes in the barriers, the diffusion of antibody through them should considerably reduce the rate of the reaction, and it was suggested that the fact that the amount of antibody adsorbed on Formvar screens was the same whether the antiserum was in contact with the slide for 15 or 40 minutes is evidence that holes are not significant.

A rough calculation indicates, however, that the time that would be required for the antibody to diffuse through holes in the barrier films is small, and even if the only mechanism by which antibody and antigen could come into contact were through this diffusion process (that is, if possible antigen diffusion or the development of more holes in the barriers upon antigen-antibody precipitate formation is ignored), the reaction might be over in a second or so. Fick's first law of diffusion is given by $dm/dt = -DA \, dc/dx$, where dm/dt is the mass of material of diffusion constant D crossing the boundary of area A in the time dt , and dc/dx is the concentration gradient in the x direction across this boundary. Let us suppose the barrier thickness is 100 Å, and the concentration of the antibody solution above the barrier is 10^{-4} gm. per cm.³. The concentration gradient of antibody across the barrier is then 10^2 gm. per cm.⁴, which we will assume remains constant during the diffusion process, since the antibody in the solution above the barrier film is present in large excess, and antibody that might diffuse through the film would be adsorbed. The diffusion constant of antibody in phosphate buffer is about 4×10^{-7} cm.² per second at room temperature. If we now assume that some small fraction of the barrier film, say 0.01 of its total surface area, has holes large enough to permit antibody to pass, and substitute these numbers into Fick's equation, $dm/dt \sim 4 \times 10^{-7} \sigma$ gm. per sec., where σ is the area of the antigen film covered by the screen. Now the four layers of antigen on the surface are equivalent to about $4 \times 10^{-7} \sigma$ gm. In other words, in such a hypothetical case, in one second an amount of

antibody equivalent to the amount of antigen on the slide would diffuse through the holes in the barrier film.

This calculation is no doubt crude, and it is presented only to indicate that the antigen-antibody reaction through holes in the barrier films might very well be completed in a time considerably less than 15 minutes, even if no other factor than antibody diffusion is considered. As a result of this, of course, no difference in antibody adsorption would have been observed at the end of 15 or 40 minutes.

On the other hand, Rothen attempted to prove that diffusion of protein through the screens was not occurring. Slides were prepared on which antibody was adsorbed directly on bovine serum albumin layers, and some of these were then coated with thin Formvar films. These two types of slides were then washed with a 5 to 10 per cent sodium chloride solution for some time. The slides with the Formvar coats did not change in thickness, while the unprotected ones suffered a considerable loss in thickness. These results were interpreted to indicate that antibody molecules could not diffuse through Formvar screens. It is possible, however, that under these conditions the salt dissociation of the antigen-antibody precipitates produced relatively large aggregates of antigen and antibody only partially solubilized, and that these were not able to diffuse through the holes in the Formvar films.

Experiments were performed by Rothen with the protamine insulin reaction to demonstrate that barium stearate and Formvar screens were effective screens for the reaction. A double layer of barium stearate covering 10 Å of the protamine clupein sulfate cut down the insulin adsorption completely, whereas 220 Å of insulin could be adsorbed directly on the protamine. However, Formvar screens 17 and 30 Å thick permitted 120 and 40 Å of insulin to be adsorbed, respectively, while for screens thicker than 50 Å no specific adsorption took place.

It is our opinion that the barium stearate screens may not be inert barriers in these experiments, but may rather react with the protamine film. Although the details of the reaction mechanism are not completely clear, it seems probable that the insulin and protamine react because the 2 molecules are oppositely charged in the physiological pH range, and attract each other by Coulomb forces. Although the protamine may originally be in the form of a sulfate, the insolubility of the protamine insulinate causes the displacement of a number of the sulfate groups from the protamine in favor of acidic groups on the insulin. Now Langmuir and Schaefer (16) have shown that between pH 7 to 10 from 40 to 5 per cent, respectively, of the carboxyl groups of the barium stearate film are free; that is, are not bound to barium ions. These free carboxyl groups might react with those groups on the protamine molecules that are avail-

able for reaction with insulin. In the process the structure of the barium stearate film might be affected sufficiently, and the reactive groups on the protamine irreversibly bound to an extent large enough so that insulin is no longer adsorbed. On the other hand, the fact that Formvar films permitted some insulin adsorption is significant.

Another set of results, dealing with the absolute amount of antibody adsorbed directly on antigen layers, is cited to indicate that long range forces operate in immunologic reactions. An extreme example of such results follows. On optical gages of octadecylamine, a layer of 700 Å of antibody was adsorbed after a long period of time on 5 Å of pneumococcus type III polysaccharide, an amount much larger than would be expected if the polysaccharide film were truly immobile and rigid and only short range forces were operating between the antigen and antibody molecules. As Rothen pointed out subsequently (23), however, studies reported by Clowes (6) indicated that up to 450 Å of insulin could be adsorbed on layers of protamine only 30 to 50 Å thick. Since this system probably reacts by a short range mechanism, such effects are therefore no indication of specific long range forces. Perhaps after the first layers of protein are specifically adsorbed on the substrate molecules, relatively non-specific polarization forces cause the immobilization of subsequent layers. It should also be pointed out that, under the influence of the forces between the reacting molecules, the layers of the substance initially deposited on the surface might not remain intact. In the experiments, for example, in which antibody was adsorbed directly on the pneumococcus polysaccharide, with no intervening barriers present, reaction with antibody might have considerably disrupted the antigen layers, dispersing some of the polysaccharide throughout the antibody. This would have permitted more antibody to be immobilized than might have been anticipated with a rigid film of antigen.

In view of this discussion, we do not consider it proved that holes in the barrier films are either absent or ineffectual. On the other hand, our experimental evidence seems to require that these holes exist. The fact that films of materials of such similar chemical constitution as the cellulose derivatives exhibit quite different screening properties, and that screens of the same polymer, ethyl cellulose, cast from two different solvents, behave very differently, indicates that it is the physical structure of these films that largely determines their screening action. In accord with this conclusion is the very marked correlation between films which exhibit the best film properties and those which inhibit antibody adsorption the most strongly. Silica films, whose surfaces are probably more uniform than those of polymers, and ethyl cellulose films cast from ethylene chloride solution, which are superior to other polymer films in stripping metal

replicas from various surfaces, are both very effective inhibitors of the antibody adsorption. These results imply that holes in the barrier films do exist, and that better films have fewer of them in a given film thickness.

The presence of distinct aggregates, which are observed in the electron microscope when the antibody is adsorbed on top of Formvar-coated layers of bovine serum albumin, also strongly suggests that antibody and antigen react through holes in the screens. These aggregates are not artifacts caused by the treatment the surfaces undergo; that is, they are not due to the Formvar itself, to non-specific aggregates in the antiserum, nor to the preparation of the chromium replica of the surface. Nor is it possible to account for them as the products of some kind of surface forces between the antibody and Formvar, since the Formvar surface is completely wet by the solution of antiserum and particularly since the aggregates on barium stearate screens were much smaller. It appears most likely, therefore, that these aggregates are antigen-antibody precipitates which have grown about holes in the Formvar films, perhaps enlarging these holes in the process.

In studying enzymatic reactions Rothen found that a screen of Formvar 500 Å thick made in two separate steps could prevent the enzymatic action of trypsin on underlying bovine serum albumin layers, while it took a single Formvar film of about 1000 Å to do the same. He ascribed this to a diminished permeability of the buffer ions of the enzyme solution when the blankets were made in two steps. An obvious explanation on the basis of the hole hypothesis is that the holes in the two separately formed films have a limited probability of coinciding to form continuous channels when the films are placed together, thus diminishing the effective number of holes below that found with single films of comparable or greater thickness.

In the light of our experiments and the information available, we suggest that the mechanism of these reactions of proteins in thin films is as follows. The bovine serum albumin layers on the slide are not uniform monolayers, but instead are quite irregular. Up to a certain point, then, the more layers of bovine serum albumin deposited on the slide, the more antigen is exposed to the action of antibody, and the more antibody is adsorbed.

Because of the nature of thin films of the type of barium stearate, and of polymers, a certain proportion of holes may be expected to be formed in them, whatever the nature of the surface on which they are deposited. Karush and Siegel (13) suggested that the roughness of the surface of the antigen layers might be responsible for the production of holes in the screens. Experiments performed by Rothen, however, are not in accord with this suggestion. In these experiments, Formvar screens were first

cast on polished glass surfaces, were floated off onto water, were then deposited on the antigen layers, and subsequently were found to permit the same antibody adsorption as Formvar films of the same thickness cast directly on the antigen layers. Moreover, the fact that enzyme activity occurs through Formvar blankets of 500 to 1000 Å thick, dimensions several times those of the largest surface irregularities of the bovine serum albumin films, also requires further explanation. We are of the opinion, therefore, that the presence of holes is an intrinsic property of these films, and that the roughness of the underlying surface is of only secondary importance to their formation. The thicker the screen, the fewer the holes running continuously through it, and the more effective a barrier it becomes. Films of silica presumably have considerably fewer holes than those of Formvar for a given film thickness, and hence inhibit antibody adsorption in much smaller thicknesses. The strength of a film, and its resistance to the strains produced by the growth of antigen-antibody precipitates, probably is of importance as well.

The detailed mechanism by which antigen and antibody come into contact through the holes in the barrier films may be complicated. Whether the antigen diffuses through or protrudes through these holes, whether it is the antibody which diffuses through them to the underlying antigen layers, or whether combinations of these and other factors come into play is at present a matter for conjecture. In the case of the enzymatic reaction of trypsin with bovine serum albumin layers, which is observed to occur through Formvar screens 500 to 1000 Å thick, we would presume that the enzyme diffuses through holes in the screens to the substrate below. One might object that Formvar films 500 to 1000 Å thick would be expected to have very few holes running continuously through them. As Rothen has noted, however, a single enzyme molecule diffusing through a blanket might damage an extensive area of the underlying substrate film. There need not be many holes, then, to account for the enzymatic activities observed. In the electron microscope we have observed that Formvar films in this range of thickness do have occasional holes running through the films which might account for these effects.

We believe this interpretation of the experimental results obtained by Rothen is self-consistent, suffices to explain the major features of these results in simple terms, and is in accord with our own experimental observations as well. On the other hand, we consider it difficult to interpret some of our results in terms of the hypothesis of long range forces. Our conclusion is, therefore, that it is by no means necessary to invoke the existence of long range forces to account for these experimental results.

In view of these considerations, and of the considerable weight of independent evidence favoring the existence of short range interactions in

immunologic and enzymatic reactions, we feel that it is unlikely that specific long range forces operate in these reactions.

SUMMARY

By studying the reactions of proteins in thin films, Rothen has obtained experimental results which he has suggested indicate that specific long range forces operate in immunologic and enzymatic reactions. He has found that antigen and antibody, and enzyme and substrate molecules, can react despite apparently being separated by thin inert barriers. We have obtained evidence by (a) investigating the behavior of certain cellulose derivatives and silica films as inert barriers, (b) by an electron microscopic examination of various surfaces involved in these experiments, and (c) by a study of the reaction in thin films of hapten-conjugated protein antigens and antibodies directed specifically against the hapten, that strongly suggests that holes in the barrier films, permitting the macromolecules to come into contact and react, are primarily responsible for the effects observed. Rothen's arguments against the existence of these holes are discussed and are considered inconclusive.

In view of this alternative explanation for the results obtained by Rothen, and of the considerable weight of evidence which seems to indicate that short range mechanisms are of primary significance in immunologic and enzymatic reactions, it appears unlikely that specific long range forces operate in these reactions.

The author is grateful to Professor Linus Pauling for suggesting this research and for his continued help and advice. The electron micrographs were taken by Professor R. F. Baker, of the University of Southern California, and Dr. B. Henke, of this Institute, and some of the specimens were prepared by Mr. R. F. Petzold; the author is indebted to them for their assistance.

BIBLIOGRAPHY

1. Bergmann, M., and Fruton, J., *Advances in Enzymol.*, **1**, 63 (1941).
2. Blodgett, K. B., and Langmuir, I., *Phys. Rev.*, **51**, 964 (1937).
3. Campbell, D. H., OEMcmr-153, Chemically modified proteins as blood substitutes, Pasadena (1946).
4. Campbell, D. H., Blaker, R. H., and Pardee, A. B., *J. Am. Chem. Soc.*, **70**, 2496 (1948).
5. Chance, B., *Arch. Biochem.*, **21**, 416 (1949).
6. Clowes, G. H. A., in Moulton, F. R., Recent advances in surface chemistry and chemical physics, Lancaster, 61 (1939).
7. Epstein, H., Thesis, University of Michigan (1949).
8. Germer, L. H., in Moulton (6), 47 (1939).
9. Haurowitz, F., *J. Immunol.*, **43**, 331 (1942).

10. Heidenreich, R. D., and Peck, V. G., *J. Appl. Phys.*, **14**, 23 (1943).
11. Iball, J., *Science*, **109**, 18 (1949).
12. Kabat, E., *Ann. Rev. Biochem.*, **15**, 505 (1946).
13. Karush, F., and Siegel, B. M., *Science*, **108**, 107 (1948).
14. Keilin, D., and Mann, T., *Proc. Roy. Soc. London, Series B*, **122**, 119 (1937).
15. Landsteiner, K., *The specificity of serological reactions*, Cambridge, revised edition (1947).
16. Langmuir, I., and Schaefer, V. J., *J. Am. Chem. Soc.*, **58**, 284 (1936).
17. Langmuir, I., and Schaefer, V. J., *Chem. Rev.*, **24**, 181 (1939).
18. Michaelis, L., and Menten, M. L., *Biochem. Z.*, **49**, 333 (1913).
19. Pauling, L., *Chem. and Ind.*, suppl. 1 (1948). Presented at the XIth International Congress of Pure and Applied Chemistry, London, July 17-24 (1947).
20. Pauling, L., Pressman, D., and Campbell, D. H., *Physiol. Rev.*, **23**, 203 (1943).
21. Pressman, D., Bryden, J. H., and Pauling, L., *J. Am. Chem. Soc.*, **70**, 1352 (1948).
22. Rothen, A., *Rev. Scient. Instruments*, **16**, 26 (1945).
23. Rothen, A., *Advances in Protein Chem.*, **3**, 123 (1947).
24. Rothen, A., *J. Biol. Chem.*, **168**, 75 (1947).
25. Rothen, A., *J. Am. Chem. Soc.*, **70**, 2732 (1948).
26. Singer, S. J., and Petzold, R. F., *J. Appl. Phys.*, in press.
27. Smith, E. L., *Proc. Nat. Acad. Sc.*, **35**, 80 (1949).
28. Spurlin, H. M., Martin, A. F., and Tennant, H. G., *J. Polymer Sc.*, **1**, 63 (1946).
29. Williams, R. C., and Backus, R. C., *J. Appl. Phys.*, **20**, 98 (1949).

EXPLANATION OF PLATES

PLATE 1

FIG. 1. Electron micrographs of replicas of the surfaces of (a) a film, approximately 1000 Å thick, of aluminum on glass; (b) four layers of bovine serum albumin on a uranyl acetate-conditioned optical gage of five layers of barium stearate on surface (a); (c) 120 Å of antibody on five layers of bovine serum albumin on the conditioned slide; (d) a 100 Å-thick Formvar film on five layers of bovine serum albumin on the conditioned slide; and (e) 90 Å of antibody on two layers of barium stearate deposited on five layers of bovine serum albumin on the conditioned slide. The scale marks represent 1 μ , and all the pictures are at the same magnification.

PLATE 2

FIG. 2. Electron micrograph of a replica of the surface of 100 Å of antibody adsorbed on a 50 Å-thick film of Formvar on five layers of bovine serum albumin on a conditioned slide. The scale mark represents 1 μ , and the picture is at the same magnification as those of Fig. 1.

a



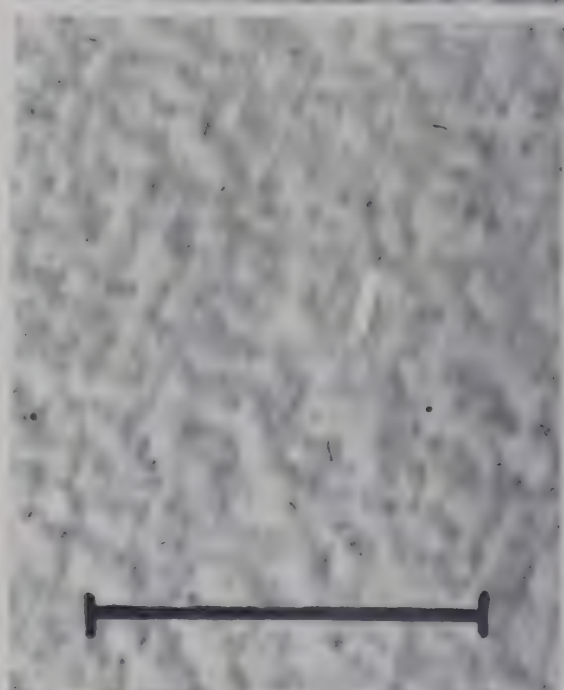
b



c

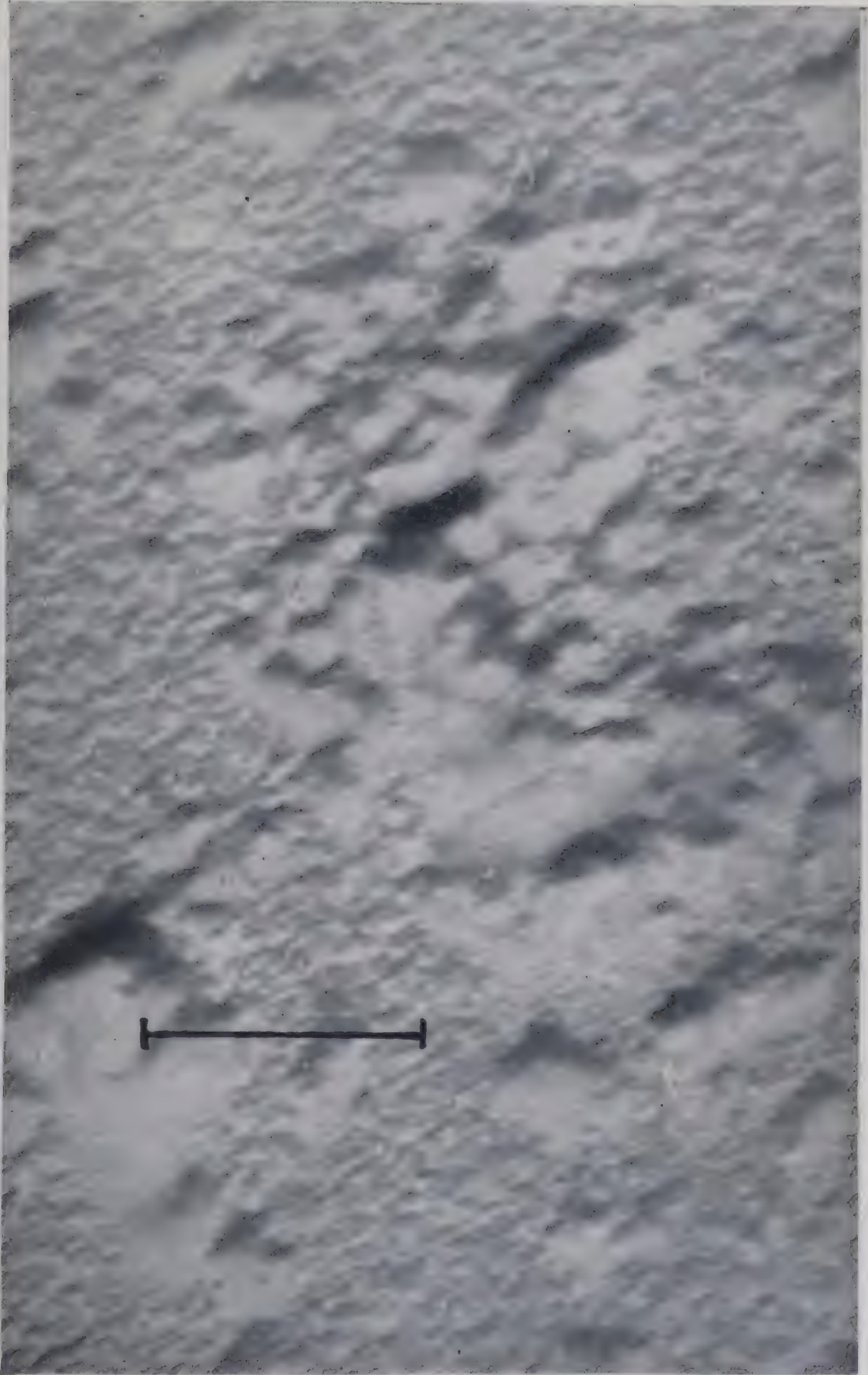


d



e





(Singer: Immunologic and enzymatic reactions)

THE CATALYTIC FUNCTION OF COENZYME A IN CITRIC ACID SYNTHESIS*

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Coenzyme A was first recognized as a component of enzymatic acetylation reactions (1, 2). Various observations, however, suggested early that this was merely a side function and that the main function of the coenzyme in cellular metabolism remained to be discovered. Most cellular pantothenic acid is present as coenzyme A (3, 4) in animal tissues as well as in microorganisms and plants. The function in acetylation, therefore, appeared not sufficient to warrant such a general presence of the coenzyme. On the other hand, the participation of a pantothenic acid derivative in the acetylation reaction tended to give new emphasis to the earlier seemingly isolated observation which showed pantothenic acid to be essential for the metabolism of pyruvate in *Proteus morganii* (5, 6). The degradation of pyruvate up to the acetate stage seemed sufficiently covered by the group of thiamine pyrophosphate enzymes. Therefore, appearance of coenzyme A as a catalytic factor in acetylation made it very likely that this coenzyme was operating in the next stage in pyruvate metabolism; namely, the feeding of the acetyl radical into the citric acid cycle, and presumably into other synthetic processes. Some indications for such an interpretation had already appeared in the work of Hills (6). Earlier work with yeast by Pratt and Williams (7) had given some hints for involvement of pantothenic acid in carbohydrate metabolism.

At first our efforts to connect coenzyme A with definite enzymatic phases of the indicated type were not very successful. The pantothenic acid effect of Dorfman *et al.* (5) and of Hills (6) in *Proteus morganii* could be traced in an over-all manner to a cellular production of coenzyme A, which thus was linked to pyruvate metabolism (8). In animal tissues, furthermore, the involvement of coenzyme A in pyruvate utilization was shown convincingly by Olson and Kaplan (9). They found with tissue slices of ducks at various stages of pantothenic acid deficiency a rather striking proportionality between the rate of pyruvate utilization and the coenzyme A content of the tissue. Yet a further breakdown towards the accurate point of action remained still to be done. In the meantime, the finding of Soodak and Lipmann (10) that an enzymatic condensation of

* This work was aided by a grant from the Commonwealth Fund.

acetate plus adenosine triphosphate (ATP) to acetoacetate was a coenzyme A-catalyzed reaction seemed to indicate that we were advancing in the right direction.

So far a study of reactions involving acetate itself rather than its precursors had given most important information on coenzyme A function. It was decided, therefore, to turn to the exploration of acetate oxidation in yeast which is known to proceed through the citric acid cycle (11). A preliminary report on the implication of coenzyme A in acetate and carbohydrate oxidation in yeast appeared previously (12). These and further experiments will be reported here in more detail.

Materials and Methods

Cultures Used—The majority of the respiratory experiments were done with *Saccharomyces cerevisiae*, strain LK2G12, obtained from Dr. John Reiner of Washington University. The essential observations were checked with other strains of *S. cerevisiae*, namely Gebrüder Mayer, ATCC 2335, as well as a strain of *Saccharomyces carlsbergensis*.

Because of the large quantity of cells necessary for the production of a cell-free extract for the study of citric acid synthesis, ordinary fresh bakers' yeast obtained from the Federal Yeast Corporation was used.

Escherichia coli 26, obtained from Dr. C. H. Werkman of Iowa State College, was used in the study of citric acid synthesis.

Methods for Growing Cells—For the respiration studies, pantothenate-deficient yeast was obtained by growing the cells for 18 to 24 hours at 30° under vigorous aeration in the basal medium of Sarett and Cheldelin (13) with from 3 to 10 γ per liter of pantothenic acid. "Normal" cells were obtained in two ways; *i.e.*, by growth in this basal medium with an excess of pantothenic acid, *e.g.* 2 mg. per liter, or by growth in a medium containing malt extract, yeast extract, glucose, and phosphate.

Respiratory Experiments—For the respiration studies, conventional Warburg methods were used. Respiratory quotients were obtained by the indirect method (14). The cell suspensions used were grown as described in the previous paragraph, harvested by centrifugation, washed twice with distilled water, and made up to a definite turbidity as measured with the Klett photocolormeter. An approximation of the dry weight of such a suspension can be made by referring to a previously calibrated standard curve prepared with suspensions of known dry weight. The actual dry weights of the suspensions were obtained by drying known aliquots at 110° overnight. Usually 4 to 8 mg. of dry weight of cells were added to each 18 ml. Warburg vessel. The total fluid volume was 3.0 ml. The buffer was 0.06 M KH_2PO_4 . When necessary, 0.2 ml. of 5 N NaOH was added to the inset to absorb carbon dioxide.

Preparation of Cell-Free Extracts for Citrate Synthesis—Large quantities of *E. coli* cells were prepared by growing the cells in 20 liter bottles containing 15 liters of medium of the following composition: 1 per cent glucose, 0.4 per cent yeast extract (Difco), 0.4 per cent peptone (Bacto), 0.8 per cent K_2HPO_4 , and 10 per cent tap water. The cells were grown for 18 hours at room temperature with vigorous aeration, and were harvested with the Sharples supercentrifuge, washed twice with distilled water, and then dried over P_2O_5 *in vacuo*.

For the preparation of cell-free extracts such dry preparations of *E. coli* were used, whereas for the yeast studies, fresh suspensions were employed. In each case, however, the essential procedure was the same. The dried cells were rubbed up in 0.02 M sodium bicarbonate solution to make a 10 per cent suspension, and, in the case of fresh yeast, a 50 per cent (wet weight) suspension was prepared. 6 or 7 ml. of this suspension were mixed with 7 to 10 gm. of glass beads (Ballotini, 0.1 mm. in diameter) and shaken for 15 minutes in a 30 ml. cup which is fastened at the end of an electromagnetically operated tuning fork. This apparatus was obtained from H. Mickle of Hampton, Middlesex, England, and was suggested for our use by Dr. D. Herbert of the Lister Institute, London, in a private communication. After such treatment, the suspension was centrifuged at 3000 R.P.M. to remove the glass and then recentrifuged at 12,000 R.P.M. for 20 minutes to remove the unbroken cells and cellular debris. The resulting supernatant was a clear, straw-yellow, somewhat viscous fluid. Further purification and concentration of the enzyme were achieved by fractionating with ammonium sulfate and taking the fraction between 35 and 70 per cent saturation.

Analytical Methods—Coenzyme A was measured by the method of Kaplan and Lipmann (3), acetate by the method of Soodak and Lipmann (15), and citric acid by the method of Krebs and Eggleston (16) modified by autoclaving at 15 pounds for 25 minutes in 3 N H_2SO_4 to remove oxalacetic acid. Acetyl phosphate was determined by the hydroxamic acid method of Lipmann and Tuttle (17). Pyruvate was determined by the method of Friedemann and Haugen (18).

Materials Used—Oxalacetic acid was prepared according to the method of Lipmann and Greene (19); lithium acetyl phosphate according to the method of Lipmann and Tuttle (20). ATP was the sodium salt obtainable from Rohm and Haas. Coenzyme A was prepared by the method used in this laboratory (21) and had an estimated purity of 25 per cent.

Effect of Pantothenic Acid on Metabolism of Resting Yeast Cells

Preliminary experiments which eventually led to the adoption of a two-stage procedure will first be discussed. In the first stage the coenzyme is

synthesized from pantothenic acid, and in the second separate stage the metabolite is tested on the enriched yeast. This method promises to be applicable in analogous situations with other vitamins as well as in obtaining distinction between effects, for example of antimetabolites, on either synthesis or metabolic action of the coenzyme (22).

When the addition of pantothenic acid to deficient suspensions of acetate-respiring yeast was first tried, little, or at best unconvincing, stimu-

TABLE I
Comparison of Oxidation of Acetate by Pantothenic Acid-Deficient Gebrüder Mayer Yeast

Time	Acetate		
	No pantothenic acid	Pantothenic acid	Per cent increase
hr.	$\mu\text{l. O}_2$	$\mu\text{l. O}_2$	
1st.....	19.3	18.9	0
2nd.....	21.9	21.4	0
3rd.....	14.3	17.3	21

Each vessel contained 5 mg. (dry weight) of cells in 0.05 M KH_2PO_4 with 25 μM of acetate in a final volume of 3.0 ml. The cells were equilibrated 15 minutes with 100 γ of pantothenic acid in the main compartment before tipping in the substrates.

TABLE II
Comparison of Respiration of Acetate and Respiration and Fermentation of Glucose by Normal and Pantothenic Acid-Deficient Yeast

	Normal		Deficient	
	Q_{O_2}	Q_{CO_2}	Q_{O_2}	Q_{CO_2}
Glucose.....	44	200	13	215
Acetate.....	35		5.0	

4.4 mg. of cells in 0.06 M KH_2PO_4 with 0.025 M glucose or 0.025 M acetate.

lation was observed. This is shown in Table I. However, when the respiratory quotients of normal and deficient yeast were compared for various substrates including acetate, deficient yeast gave much lower respiratory rates, as is shown in Table II. In contrast, fermentation was quite unaffected by pantothenic acid deficiency. A clue to this somewhat paradoxical behavior was found when the effect of pantothenic acid was studied with deficient yeast, respiring glucose. It may be seen in Table III that stimulation increases steadily with time and becomes quite considerable in the 3rd hour. A slight indication for increase with time may already be found in the experiment in Table I with acetate.

Since this yeast has a strong aerobic fermentation, most of the glucose actually disappears from the reaction medium during the earlier part of the experiment. In the later part, the yeast therefore mainly respire alcohol, and it seemed possible that the increasing effect on respiration might indicate a selective effect of pantothenic acid on alcohol oxidation. Therefore alcohol-respiring yeast was tested directly for an effect of pantothenic acid, but no such effect was found. Like acetate respiration, alcohol res-

TABLE III

Comparison of Oxidation of Glucose by Pantothenic Acid-Deficient Gebrüder Mayer Yeast

Time	Glucose		
	No pantothenic acid	Pantothenic acid	Per cent increase
min.	$\mu\text{l. O}_2$	$\mu\text{l. O}_2$	
0- 60	39.2	46	17
60-120	76.8	97	26
120-160	66	101	53

Conditions as in Table I except that, instead of acetate, 25 μM of glucose were added.

TABLE IV

Effect of Added Pantothenic Acid on Oxygen Consumption with Alcohol by Pantothenic Acid-Deficient Gebrüder Mayer Yeast

Time	No substrate	Alcohol	
		No pantothenic acid	Pantothenic acid
min.	$\mu\text{l. O}_2$	$\mu\text{l. O}_2$	$\mu\text{l. O}_2$
0- 60	19	26	25
60-120	20	47	48

Conditions as in Table I, with alcohol as substrate instead of acetate.

piration of deficient yeast did not respond directly to added pantothenic acid (Table IV).

It was suspected from these observations that synthesis of coenzyme A from pantothenic acid requires a considerable input of energy and therefore occurs reliably only with yeast that actively respire glucose. This was confirmed by determination of coenzyme A content in deficient yeast samples after aeration for 1 to 1½ hours in a glucose-phosphate medium with and without pantothenic acid. After such treatment the pantothenic acid sample showed a 2- to 3-fold increase in coenzyme A content. On the average, the deficient yeast contained 150 units per gm. of coenzyme

A, and, after incubation with pantothenic acid, glucose, and phosphate, the coenzyme A content increased to around 400 units per gm.

High metabolic requirements for coenzyme A synthesis were earlier indicated on different material by the observations by Olson and Kaplan (8), who found that only slight synthesis of coenzyme occurred with deficient liver slices when pantothenic acid was added to the medium. On injection of pantothenic acid into the whole deficient animal, however, coenzyme A was very rapidly synthesized in the liver.

As a result of this preliminary observation, it appeared most appropriate for the study of metabolic effects of coenzyme A to use coenzyme A-enriched samples obtained by preincubation in the glucose-phosphate medium. An example of the routine procedure adopted is included.

	ml.	ml.
Yeast suspension.....	8.0	8.0
Glucose 0.5 M.....	0.2	0.2
Pantothenate (1 mg. per ml.).....	0.1	
KH ₂ PO ₄ 0.1 M.....	3.0	3.0
H ₂ O.....		0.1

The flasks are aerated by bubbling air through the suspension for 1 hour at 30°. Then both suspensions were centrifuged, washed twice, and re-suspended in 8.0 ml. of 0.1 M primary phosphate solution.

The yeast suspension had 2.8 mg. per ml. of dry weight of cells, which did not increase in turbidity during the aeration period. The initial coenzyme A concentration of the suspension was 100 units per gm. and after the aeration period 370 units per gm. for Suspension 1 and 110 units per gm. for Suspension 2. In this procedure it is therefore possible to enrich a deficient suspension and have the enriched sample and its deficient counterpart available for metabolic or respiratory studies which could be conducted in the second stage in the absence of the particular essential metabolite. In this experimental set up, no pantothenic acid is present in the medium in the final test. The yeast samples preincubated with and without vitamin will be referred to as coenzyme A-rich and coenzyme A-poor yeast, respectively.

In this procedure the involvement of coenzyme A in acetate oxidation could readily be shown. Consistently the respiratory rate was increased in the coenzyme A-rich sample to twice or more than that found in the coenzyme A-poor sample. The experiment presented in Fig. 1 shows, in addition, that only on preincubation with pantothenic acid but not with niacin or thiamine, was an increase in acetate oxidation obtained. A further confirmation was found by a determination of acetate disappearance in addition to oxygen consumption. Acetate was determined by the enzymatic method of Soodak and Lipmann (15), for which only a fraction

of the amount used in a single Warburg vessel was needed. The data given in Table V show that the rate of oxygen and acetate consumption is proportionally lowered in coenzyme A deficiency, while the oxygen to acetate ratio remains the same in both. 2 moles of oxygen were used per mole of acetate, which indicates complete oxidation in both cases. It is

EFFECT OF CoA ON ACETATE OXIDATION IN YEAST

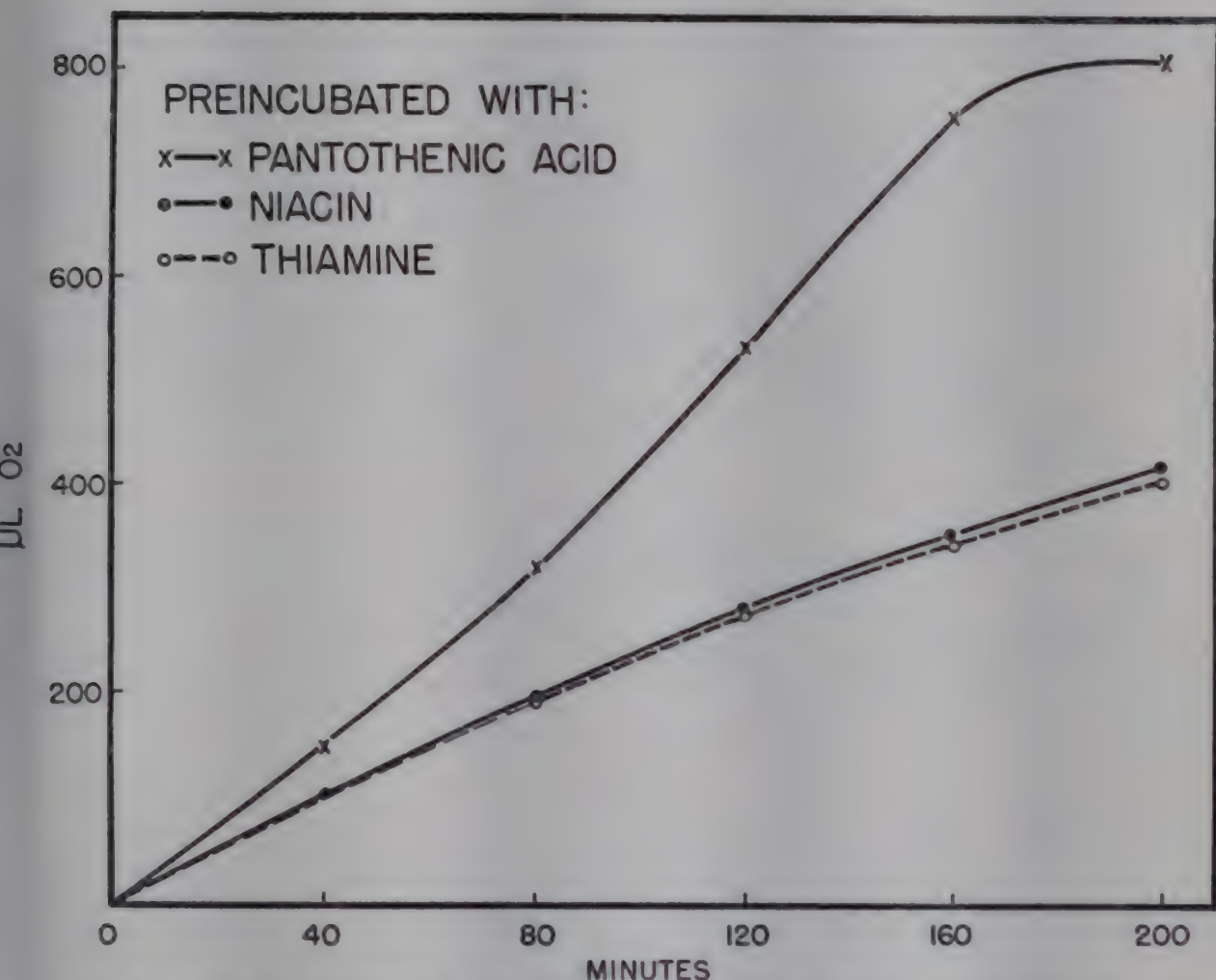


FIG. 1. Effect of coenzyme A on acetate oxidation in yeast. Pantothenate-deficient yeast was preincubated in glucose-phosphate medium with 50 γ of pantothenate, 100 γ of thiamine, and 100 γ of niacin in separate flasks. 5.6 mg. of dry weight of each suspension were added to individual Warburg vessels. Total fluid volume 3.0 ml. of 0.06 M KH_2PO_4 , and 0.01 M acetic acid; temperature 37°; gas phase air.

therefore concluded that it is the rate of the primary attack on acetate which is depressed in coenzyme A-poor yeast. In other words, coenzyme A should be involved in the initial condensation of acetate and oxalacetate, through which the citric acid cycle leads to complete oxidation.

The oxidation of ethanol was studied in some detail. Ethanol is primarily oxidized to acetate. With a block of acetate utilization, the ace-

TABLE V

Comparison between Oxygen Consumption and Acetate Utilization by High and Low Coenzyme A Yeasts

Time	Low coenzyme A (130 units per gm.)			High coenzyme A (370 units per gm.)		
	O ₂ used	HAc used	$\frac{O_2}{HAc}$	O ₂ used	HAc used	$\frac{O_2}{HAc}$
min.	μM	μM		μM	μM	
50	6.15	3.07	2.0	7.9	3.95	2.0
100	12.6	6.5	2.0	17.0	8.5	2.0
165	18.6	9.3	2.0	33.0	16.5	2.0
Theoretical for complete oxidation			2.0	2.0		

4.3 mg. of cells in 3.0 ml. of 0.06 M KH₂PO₄; 20 μM of acetic acid added.

EFFECT OF CoA ON ALCOHOL OXIDATION IN YEAST

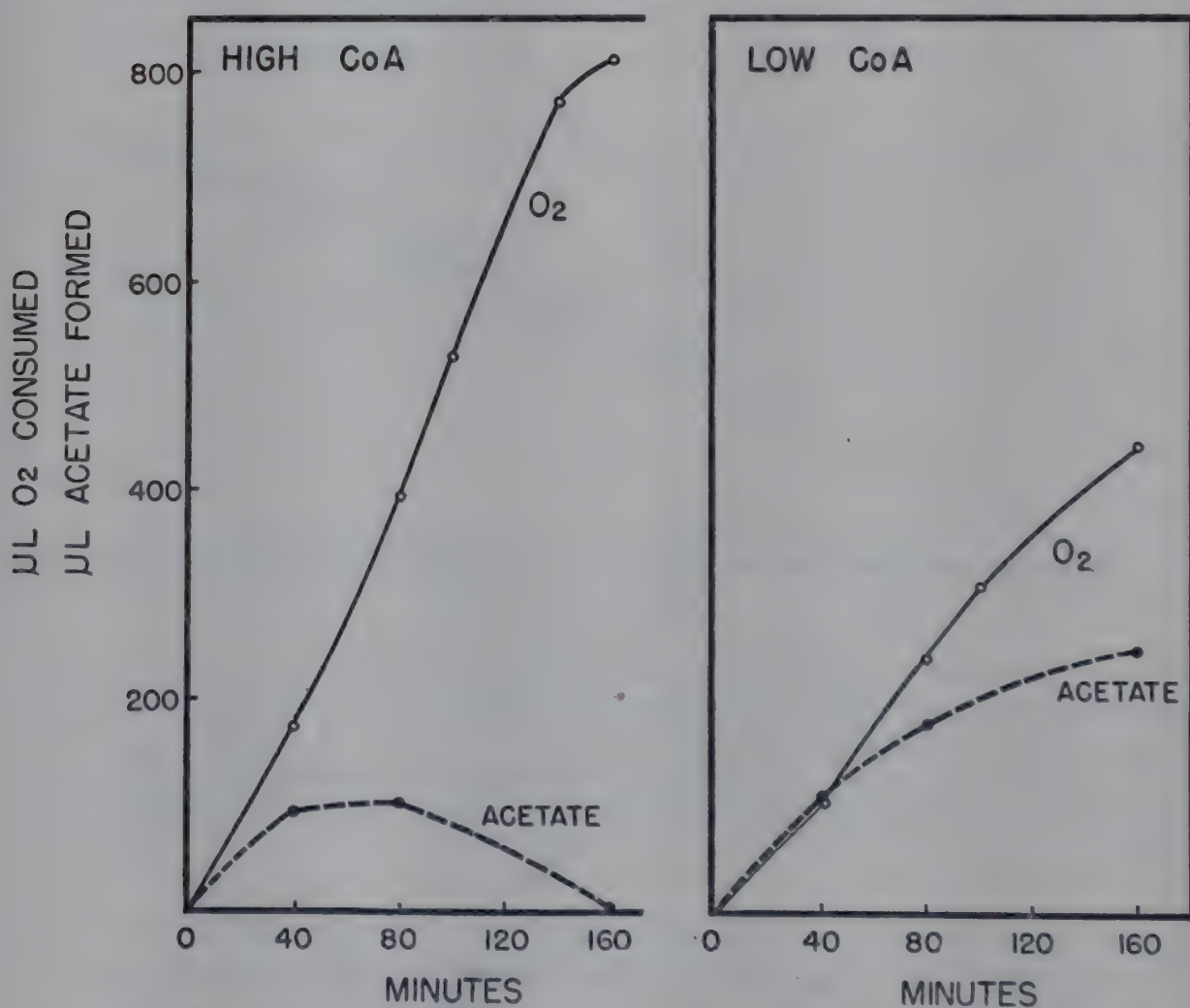


FIG. 2. Effect of coenzyme A on alcohol oxidation in yeast. Each vessel contained 4.3 mg. of dry cells in 3.0 ml. of 0.02 M KH₂PO₄; temperature 37°; gas phase air.

tate should accumulate, and furthermore the respiratory quotient should be lowered; the primary oxidation step up to acetate should be independent of coenzyme A. The results of measurements of oxygen consumption and acetate accumulation with ethanol-respiring yeast are represented in Fig. 2, and the respiratory quotients for the same experiment are recorded in Table VI.

The coenzyme A-rich yeast piles up some acetate in the beginning, cor-

TABLE VI

Comparison of Respiratory Quotient during Oxidation of Alcohol by Low and High Coenzyme A Yeast

Time	Low coenzyme A (130 units per gm.)	High coenzyme A (370 units per gm.)
<i>min.</i>	<i>R.Q.</i>	<i>R.Q.</i>
20	0.09	0.28
40	0.22	0.39
60	0.37	0.44
80	0.35	0.50
100	0.38	0.60

TABLE VII

Oxygen Consumption and Acetate Accumulation in Oxidation of Glucose by Low and High Coenzyme A Yeast

Time	Low coenzyme A	High coenzyme A
<i>min.</i>	$\mu\text{l. O}_2$	$\mu\text{l. O}_2$
20	76	79
40	175	191
60	222	310
80	260	354
Acetate found, μM	2.5	0

4.3 mg. of cells in 3.0 ml. of 0.06 M KH_2PO_4 ; 5 μM of glucose added.

responding with a low respiratory quotient in the 1st hour. After a little over 2 hours, however, the acetate has disappeared and the respiratory quotient rises towards the normal 0.66, the value at 100 minutes having gone up to 0.6. In the coenzyme A-low yeast the oxygen consumption is low. Acetate accumulates steadily and does not disappear in the later stage of the experiment. The respiratory quotient rises much more slowly and after 1 hour reaches apparently a maximum of 0.38. A more thorough comparison of the values (23), not given here in detail, shows rather accurately that the two dehydrogenation steps from ethanol to acetate are

normal in the coenzyme A-deficient yeast, while it is the utilization of acetate which is inhibited.

It has been shown by Weinhouse and Millington (11) that yeast respire glucose through acetate over the citric acid cycle. Glucose oxidation in

TABLE VIII
Citric Acid Synthesis in Yeast Extract

Additions	Citric acid synthesized per ml. extract
	μM
None.....	0*
".....	0.41
ATP.....	0.66
" coenzyme A.....	1.39
Acetyl phosphate.....	0.45
" " coenzyme A.....	0.45

All the tubes contained sodium acetate 0.02 M, sodium oxalacetate 0.025 M, NaHCO_3 0.016 M, cysteine 0.01 M, MgCl_2 0.01 M. The additions had the following concentrations: ATP 0.02 M, lithium acetyl phosphate 0.02 M, coenzyme A 12.5 units. The enzyme was an extract prepared from fresh bakers' yeast by shaking with Ballotini and centrifuging at 11,000 R.P.M.

* Oxalacetate left out of this tube.

TABLE IX
Citric Acid Synthesis in Purified Yeast System

Additions	Citrate per ml. extract
	μM
Acetate.....	0
" oxalacetate.....	0
" " ATP.....	0.23
" " " coenzyme A.....	1.68

Each tube contained cysteine 0.01 M, NaHCO_3 0.016 M, MgCl_2 0.01 M. The concentration of the additions was as follows: acetate 0.05 M, oxalacetate 0.025 M, ATP 0.02 M, Co A 12.5 units. 130 ml. of yeast juice were fractionated with ammonium sulfate between 30 and 70 per cent saturation. The precipitate of 30 per cent saturation was discarded. 90 ml. of supernatant were brought to 70 per cent saturation. The precipitate was dissolved in water and dialyzed against 0.9 per cent KCl for 18 hours in the cold; the final volume was 20 ml.

coenzyme A-deficient yeast, therefore, should also lead to an accumulation of acetate. This is shown in an experiment presented in Table VII. The deficiency, indeed, appears most pronounced in the accumulation of acetate, which is absent in the coenzyme A-rich yeast, while the respiratory rate is only slightly depressed,

Coenzyme A Effect on Citric Acid Synthesis in Cell-Free Extracts¹

Thus far experiments with living yeast cells had proved as convincingly as possible with such a system that the condensation of acetate and oxal-

TABLE X
Citric Acid Synthesis in Dialyzed Extract of E. coli

Additions	Citric acid synthesized per ml. extract
	γM
None.....	0
Acetate, ATP.....	0.23
“ “ coenzyme A.....	1.30
Acetyl phosphate.....	0.25
“ “ coenzyme A.....	4.0

All tubes contained 1.0 ml. of extract, 0.025 M oxalacetic acid, 0.016 M NaHCO_3 , 0.02 M MgCl_2 , and 0.01 M cysteine in a final volume of 2.5 ml. The concentrations of the additions were as follows: sodium acetate 0.05 M, sodium ATP 0.02 M, lithium acetyl phosphate 0.004 M, and coenzyme A 17 units.

TABLE XI
Citrate Synthesis in Fractionated E. coli Extract; Comparison of ATP and Acetyl Phosphate

	Citrate per ml. extract
	μM
5 μM ATP, coenzyme A.....	0.94
10 “ “ “ “.....	1.38
20 “ “ “ “.....	1.36
20 “ “ no coenzyme A.....	0
3.3 “ acetyl phosphate, coenzyme A.....	1.8
6.6 “ “ “ “.....	3.12
13.2 “ “ “ “.....	3.12
13.2 “ “ “ no coenzyme A.....	0

Reaction mixture, in the experiments with ATP, acetate 0.05 M, oxalacetate 0.025 M, MgCl_2 0.01 M, cysteine 0.01 M, sodium bicarbonate 0.016 M. In the experiments with acetyl phosphate the molar concentrations were the same, except that acetate was left out. The enzyme was prepared from the usual type of extract by fractionating with ammonium sulfate and taking the fraction between 35 and 70 per cent saturation.

acetate to citric acid involves coenzyme A. We then attempted preparing yeast extracts to study citric acid synthesis in a cell-free system. After

¹ When these experiments were in progress, a note by Stern and Ochoa (24) appeared reporting a coenzyme A-dependent citric acid synthesis in such an ammonium sulfate fraction of pigeon liver extract as had been used previously by Soodak and Lipmann (10) for the demonstration of coenzyme A-dependent acetoacetate synthesis.

some preliminary experimentation, reasonably active extracts were obtained by the glass bead-shaking method described earlier in this paper. Commercial bakers' yeast was used. The crude extract showed considerable blanks, but some definite effects of coenzyme A were observed. An experiment of this type is shown in Table VIII. The effect of ATP and

TABLE XII
Pyruvate Synthesis in E. coli Extracts

Additions	Pyruvate formed per ml. extract
	μM
Acetate + ATP.....	3.5
Acetyl phosphate.....	0.27

The reaction mixture contained sodium formate 0.05 M, $MgCl_2$ 0.02 M, cocarboxylase 40 γ , coenzyme A 12.5 units. The additions were added to give a final concentration: acetate 0.1 M, ATP 0.01 M, acetyl phosphate 0.01 M.

TABLE XIII
*Citrate Synthesis in Pigeon Liver Extract**

Additions	Citrate per ml. extract
	μM
None.....	0.53
Acetate, coenzyme A.....	0.56
“ ATP, coenzyme A.....	4.30
Acetyl phosphate, coenzyme A.....	0.69

The reaction mixture contained $NaHCO_3$ 0.1 M, cysteine 0.01 M, $MgCl_2$ 0.02 M, sodium oxalacetate 0.025 M, Na_2SO_4 0.04 M. The additions were made to give a final concentration as follows: sodium acetate 0.05 M, sodium ATP 0.02 M, sodium acetyl phosphate 0.035 M, and coenzyme A 12.5 units. The enzyme was prepared from an aged extract of acetone-dried pigeon liver by making a precipitation of 20 per cent saturated ammonium sulfate solution at pH 4.4. The resulting precipitate is dissolved in 0.02 M $NaHCO_3$ and dialyzed against 0.02 M $NaHCO_3$, 0.001 M cysteine, and 0.9 per cent KCl at 5° for 18 hours.

* Chou, T. C., and Lipmann, F., unpublished observations.

coenzyme A on the citric acid synthesis appears quite clearly. Acetyl phosphate could not replace ATP plus acetate. In order to bring out the coenzyme effect more convincingly, a fractionation of the yeast extract was carried out. The ammonium sulfate fraction between 30 and 70 per cent saturation contained most of the activity. In this system appreciable condensation occurred only with added coenzyme A. This is shown in Table IX. It should be mentioned that this yeast extract showed poor if any acetyl phosphate formation on addition of ATP plus acetate. These

checks were carried out with hydroxylamine added either during or after incubation.

Experiments with Extract of Dried E. coli

The preparation used here was obtained with the same strain of *E. coli* which had been used by Kaplan and Lipmann (25). In the present study the extracts were prepared by shaking the dried *E. coli* suspension with the glass bead disintegrator. It soon appeared that this extract very effectively synthesizes citrate, but, to our considerable surprise, synthetic acetyl

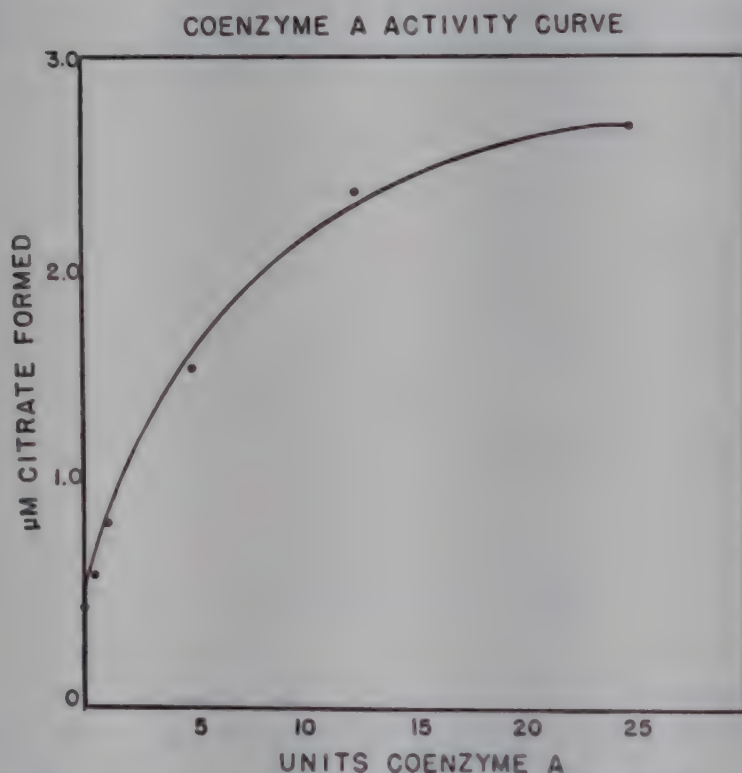


FIG. 3. Coenzyme A activity curve. The reaction mixture contained 1.0 ml. of fractionated *E. coli* extract in 2.5 ml., total volume, sodium oxalacetate 0.025 M, lithium acetyl phosphate 0.015 M, cysteine 0.01 M, $MgCl_2$ 0.01 M, phosphate buffer 0.05 M, pH 7.0; gas phase air; incubated 1 hour at 30°.

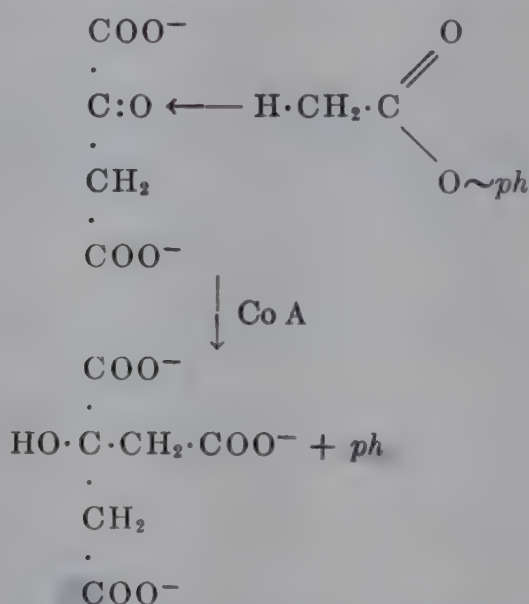
phosphate was the most potent acetyl precursor. A dialyzed *E. coli* extract was used in the experiment shown in Table X. A more detailed comparison of the effects of ATP plus acetate and acetyl phosphate was carried out with a purified system (Table XI). This brings out even more definitely the superiority of acetyl phosphate, which almost quantitatively may be converted to citric acid.

For comparison, the inactivity of synthetic acetyl phosphate in such an extract to promote pyruvate synthesis is shown in Table XII (cf. Kaplan and Lipmann (25)). Furthermore, it is noteworthy that synthetic acetyl phosphate did not synthesize citric acid in pigeon liver extract which showed considerable activity with ATP plus acetate (Table XIII).

The coenzyme A concentration-activity curve for citric acid synthesis is shown in Fig. 3. The saturation point is at about 25 units of coenzyme A per ml., which is a little higher than the concentration needed for saturation of the acetoacetate synthesis system and is considerably higher than that for sulfanilamide acetylation.

DISCUSSION

The activity of synthetic acetyl phosphate in citric acid synthesis in *E. coli* extracts deserves some attention. This activity must be considered a direct donation of acetyl because of the lower activity of ATP plus acetate, even at considerably higher absolute concentration. Furthermore,



the inactivity of synthetic acetyl phosphate in the same extract in pyruvate synthesis excludes an indirect action through phosphate donation.

The citric acid synthesis as it appears from these observations is shown in the accompanying diagram. It differs from the acetylation reaction studied so far in that it represents a reaction in which the acetate is only activated in the methyl or tail group. In all the other acetylation reactions the acetate activation occurs only, or at least partly, in the carboxyl or head part of the acetate. Acetoacetate condensation presents a special case in that it is a head to tail condensation, and it is still undecided whether a head activation is sufficient or whether both acetates, the one at the carboxyl and the other at the methyl group, have to be activated.

It seems that in a case of an exclusive methyl activation synthetic acetyl phosphate can act as acetyl precursor. However, special conditions appear required for such an activity, since yeast extract and pigeon liver extract cannot use synthetic acetyl phosphate for citric acid synthesis.²

² Recent work by Novelli, Stadtman, Chou, and Lipmann, to be published soon, has shown that extracts of *E. coli* and *Clostridium kluyveri* contain an enzyme which

SUMMARY

The synthesis of coenzyme A from pantothenic acid occurs in yeast best on aeration in a glucose-phosphate medium. With acetate or ethanol as the respiratory substrate, no or sluggish synthesis of the coenzyme occurs.

A two-step procedure for the study of metabolic effects of coenzyme A is described, consisting in a primary coenzyme A enrichment by aeration in pantothenic acid-glucose-phosphate medium. The effect of the enriched organisms on various metabolites has been studied.

Coenzyme A enrichment from 150 to around 400 units per gm. of yeast increases acetate and ethanol respiration 2- to 3-fold. With ethanol as substrate in the coenzyme A-poor yeast, an accumulation of acetate accompanied the lower respiration. This is abolished with increased coenzyme A concentration. A slighter acetate accumulation in coenzyme A-deficient yeast is also found with glucose oxidation. These results indicate involvement of coenzyme A in acetate activation for citric acid synthesis.

The catalytic function of coenzyme A in citric acid synthesis is confirmed in experiments with cell-free extract of yeast and *Escherichia coli*. In extracts of *E. coli*, synthetic acetyl phosphate may act as acetyl precursor in citric acid synthesis, preferentially to ATP plus acetate.

BIBLIOGRAPHY

1. Lipmann, F., *J. Biol. Chem.*, **160**, 173 (1945).
2. Lipmann, F., Kaplan, N. O., Novelli, G. D., Tuttle, L. C., and Guirard, B. M., *J. Biol. Chem.*, **167**, 869 (1947).
3. Kaplan, N. O., and Lipmann, F., *J. Biol. Chem.*, **174**, 37 (1948).
4. Novelli, G. D., Kaplan, N. O., and Lipmann, F., *J. Biol. Chem.*, **177**, 97 (1949).
5. Dorfman, A., Berkman, S., and Koser, S. A., *J. Biol. Chem.*, **144**, 393 (1942).
6. Hills, G. M., *Biochem. J.*, **37**, 418 (1943).
7. Pratt, E. F., and Williams, R. J., *J. Gen. Physiol.*, **22**, 637 (1939).
8. Novelli, G. D., and Lipmann, F., *Arch. Biochem.*, **14**, 23 (1947).
9. Olson, R. E., and Kaplan, N. O., *J. Biol. Chem.*, **175**, 515 (1948).
10. Soodak, M., and Lipmann, F., *J. Biol. Chem.*, **175**, 999 (1948).
11. Weinhouse, S., and Millington, R. H., *J. Am. Chem. Soc.*, **69**, 3089 (1947).
12. Novelli, G. D., and Lipmann, F., *J. Biol. Chem.*, **171**, 833 (1947).
13. Sarett, H. P., and Cheldelin, V. H., *J. Bact.*, **49**, 31 (1945).
14. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric techniques and related methods for the study of tissue metabolism*, Minneapolis (1945).

apparently converts synthetic acetyl phosphate into an active acetyl donor. Addition of microbial extracts, or fractions thereof, to acetone pigeon liver extract makes the synthetic acetyl phosphate fully reactive as acetyl donor in sulfanilamide acetylation as well as in citrate and acetoacetate synthesis. The activation of synthetic acetyl phosphate is therefore not restricted to reactions involving its methyl part. The inactivity of the synthetic compound in pyruvate synthesis with extracts of *E. coli* containing the "conversion factor" still remains to be explained.

15. Soodak, M., and Lipmann, F., *Federation Proc.*, **7**, 190 (1948).
16. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, **38**, 426 (1944).
17. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **159**, 21 (1945).
18. Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147**, 415 (1943).
19. Lipmann, F., and Greene, R., in Carter, H. E., *Biochemical preparations*, New York, **2**, in press.
20. Lipmann, F., and Tuttle, L. C., *Arch. Biochem.*, **13**, 373 (1947).
21. Lipmann, F., and Kaplan, N. O., *Federation Proc.*, **5**, 145 (1946).
22. Novelli, G. D., and Lipmann, F., *Federation Proc.*, **7**, 177 (1948).
23. Novelli, G. D., Thesis, Harvard University (1949).
24. Stern, J. R., and Ochoa, S., *J. Biol. Chem.*, **179**, 491 (1949).
25. Kaplan, N. O., and Lipmann, F., *J. Biol. Chem.*, **176**, 459 (1948).

BIOCHEMICAL STUDIES OF VIRUS REPRODUCTION

III. THE ORIGIN OF VIRUS PHOSPHORUS IN THE *ESCHERICHIA COLI* T₆ BACTERIOPHAGE SYSTEM*

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The purpose of this investigation has been to study the nature of the biological precursors of virus nucleic acid phosphorus in the *Escherichia coli* T₆r⁺ bacteriophage system. Previous communications (1, 2) have presented methods for the growth and isolation of this phage and have described the physicochemical and analytical characterization of the purified virus. In a preliminary report (3) it was shown by isotopic methods that in a chemically defined medium the medium itself is the ultimate source of 70 per cent of the P of T₆r⁺ bacteriophage, but that the remainder of the phage P is derived from host material originally present at the time of infection. This conclusion is in accord with observations of Cohen (4, 5) for bacteriophages T₂r⁺ and T₄r⁺ grown in synthetic medium. Similar results have now been obtained on T₆r⁺ bacteriophage cultured on host cells maintained in nutrient broth medium. While a substantial contribution of bacterial P to the virus progeny has been clearly established, the nature of this virus precursor has not been completely ascertained; nor has its significance been elucidated in regard to the mechanism of virus reproduction. However, experiments with host cells differentially labeled in the several phosphorus fractions indicate that bacterial nucleic acid rather than the pool of acid-soluble P is the major source of the bacterial contribution to virus P.

EXPERIMENTAL

Materials and Methods—The techniques used for the propagation, purification, and analysis of bacteriophage T₆ have previously been described (1, 2). In some experiments the phosphate concentration of the synthetic medium (1) was diminished in order to obtain greater incorporation of added P³². Bacterial concentrations were determined by turbidity measurement with the Evelyn colorimeter, with the appropriate blank and filter (660 mμ for the broth medium and 490 mμ for the synthetic medium). By

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use of a calibration curve relating colony count to turbidity, it was possible to estimate the cell count rapidly with an accuracy of 5 to 10 per cent.

The radioactive P used in these experiments was obtained from the Oak Ridge National Laboratory as a phosphate solution at pH 4.5. The original solutions were found to contain activity (in some cases as much as 30 per cent) which was not precipitable as inorganic phosphate with magnesia mixture. The solutions were therefore routinely hydrolyzed in N HCl at 100° for 15 minutes. After neutralization it was found that 99.8 per cent of the radioactive material was precipitated with the magnesia mixture.

Radioactivity was measured with an all-glass Geiger-Müller counter tube connected to a scale of 32 circuit. All measurements were made on 1 ml. liquid samples under conditions of constant geometry, yielding a direct linear relationship between concentration of P^{32} and counts. None of the aqueous solutions affected the counting rate, but counts on alcoholic solutions (containing the lipid fractions) had to be corrected by an empirically determined factor, 0.90, because of the lower density of the alcohol. In general the considerations and directions of Reid (6) were followed in the measurement of the radioactivity.

Effect of Radioactivity on Growth of E. coli and T₆ Bacteriophage—Although it is known that high levels of radioactivity (50 μ c. of P^{32} per ml.) kill resting *E. coli* cells (7), no evidence was available on the effect of P^{32} on growing bacteria. In exploratory experiments it was found that 0.05 to 10 μ c. of P^{32} per ml. failed to affect the rate of growth of *E. coli* in broth. Accordingly, in the experiments, 1 to 5 μ c. of P^{32} per ml. were added to assure the incorporation of large amounts of radioactivity in the bacteria or virus. Purified T₆ bacteriophage suspended in solutions of this activity was unaltered either in respect to infectivity or to plaque type.¹

Exchange of P³² by T₆ Bacteriophage—The possibility of exchange of phosphorus between the bacteriophage and the medium was investigated with P^{32} as a tracer. Highly purified phage, at a final concentration of 1 to 2×10^{12} particles per ml., was suspended in a sterile saline solution of $Na_2HP^{32}O_4$, containing a total radioactivity of 50 to 100 μ c., at a phosphate concentration of 0.003 M at pH 7.2. In a series of experiments the phage was incubated for from 2 to 38 hours at 37°, then sedimented in the cold at $20,000 \times g$ and resuspended in saline. After four washings the virus suspension was dialyzed for 24 hours against several changes of saline. The virus P was separated into fractions containing (1) acid-soluble P, (2) lipid P, (3) desoxyribonucleic acid (DNA P), and (4) ribonucleic acid

¹ In experiments concerning the fate of the infecting virus particle some indication was obtained of a drop in titer attributable to the high radioactivity incorporated in the bacteriophage (8).

(RNA P) plus phosphoprotein (2, 9, 10). In all these experiments the virus preparation contained radioactive P. However, in each instance the counts were almost entirely in the non-DNA P components of the phage, the activity of the phage DNA being only about 0.1 per cent that of the suspending labeled medium. The inertness of phage DNA P, as shown by the failure of exchange to occur in this fraction, makes P tracer experiments with phage feasible, provided the radioactivity of this separated fraction is followed.

Much attention was paid to the nature of the P^{32} found in the acid-soluble and ribonucleic acid fractions of the virus in the exchange experiments. A significant amount of the P^{32} taken up by the acid-soluble and RNA fractions was no longer precipitable as inorganic P by magnesia mixture. Most of the radioactivity could be removed by dialysis against N phosphate or arsenate buffer, though not against saline. The addition of fluoride and cyanide suppressed the exchange of P^{32} by the virus. The physiological significance of the phenomenon is considered dubious because the extent of exchange seemed related to the purity of the phage preparation. The smallest uptake of P^{32} from sterile inorganic medium was found for virus, giving a single boundary in the ultracentrifuge and in the electrophoresis apparatus. A similar exchange of radioactive phosphate was observed by Born, Lang, and Schramm (11) in experiments with tobacco mosaic virus and was attributed by these authors to adsorption of inorganic phosphate.

Labeled Medium As Source of Phage P—The origin of virus P was studied in synthetic lactate medium with two complementary systems: (1) unlabeled cells in medium containing P^{32} , and (2) washed labeled cells in unlabeled medium. In each instance the bacteria were infected multiply to produce a single generation of phage (1, 4, 12) and the virus harvested and purified by procedures already described (1). Bacteriophage isolated by these methods is essentially homogeneous in the ultracentrifuge and in the electrophoresis apparatus (2).

A typical protocol follows for an experiment designed to determine the contribution of labeled medium P to phage growing in unlabeled cells (Experiment I, Table I): 2 liters of synthetic lactate medium were inoculated with 10 ml. of an 18 hour subculture of *E. coli*, strain B, and the suspension incubated at 37° with aeration. When the bacterial concentration was about 7.5×10^7 cells per ml., 1 mc. of P^{32} was added and the bacteria simultaneously infected with a purified concentrate of T₆ bacteriophage at a phage to bacterium ratio of 4:1. After 15 hours incubation with aeration, the titer of the turbid lysate had reached 1.34×10^{10} virus particles per ml. The phage was purified by differential centrifugation and the virus P chemically fractionated by the methods previously mentioned.

The specific activity (counts per minute per microgram of P) was determined on aliquots of the original labeled medium and of the phage P fractions. From the data of Experiment I, Table I, it can be seen that the specific activity of the phage DNA was 76 per cent that of the medium. The results show that under these conditions the bulk of virus P is assimilated from the medium after infection, but that a significant portion, some 25 per cent, is derived from P assimilated by the host prior to infection.

A similar experiment was performed with unlabeled cells in nutrient broth medium to which $\text{NaH}_2\text{P}^{32}\text{O}_4$ had been added (Experiment II, Table I). The radioactivity of the phage DNA was 90 per cent that of the

TABLE I
*Radioactivity of T_6 Bacteriophage Grown on *E. coli* with Cells or Medium Containing P^{32}*

Experiment No.	Bacteria	Specific radioactivity, c.p.m. per γ P					Relative radioactivity*		
		Medium	Bacteria			Virus DNA	Virus DNA Medium	Virus DNA Bacterial P	Virus DNA Bacterial DNA P
			Total P	Acid-soluble P	DNA				
I	Unlabeled	(L.) 17.1	0	0	0	12.9	76		
II	"	(B.) 845†	0	0	0	668	79		
III	Labeled	(L.) 0	80.6	80.6	75.8	19.6		24	26
IV†	"	" 0	250	258	260	43.2		17	17
V	Differentially labeled	" 0	348	195	415	128		37	31

L. signifies synthetic lactate medium; B., nutrient broth.

* Ratio of specific activities $\times 100$.

† Calculated for inorganic P content of broth medium.

‡ An ultracentrifuge analysis of this phage preparation showed only one component.

medium when calculated on the basis of the ratio of the specific activity of phage DNA to specific activity of total medium P. However, when correction was made for the fact that the organic P constituents of broth comprise from 11 to 17 per cent of the total P and only inorganic P^{32} was added as a tracer, it appeared that about 79 per cent of the virus phosphorus had been assimilated from the medium. Evidence to be presented in a later section excludes the possibility that the organic P of the medium rather than the host P was the source of the unlabeled P found in the virus.

Labeled Cells As Source of Phage P—The previous experiments had suggested that bacterial P to the extent of about 25 per cent of total phage P

was utilized in the synthesis of the virus. This point was confirmed directly by infection of washed P^{32} -labeled cells in unlabeled synthetic medium (Experiments III and IV, Table I). The procedure was as before, except that the bacteria were grown in synthetic lactate medium containing either 0.5 or 1 μ c. of P^{32} per ml., but at one-half or one-fifth the usual phosphate concentration in order to obtain more efficient incorporation of the radioactivity. The bacteria were centrifuged and washed three times in saline prior to the measurement of radioactivity and P partition analysis on aliquots. In synthetic medium, but not in broth (compare below), all the P fractions of the bacteria, *i.e.* DNA, RNA, etc., had the same specific radioactivity (counts per minute per microgram of P) as did the medium. The labeled bacteria were resuspended in fresh isotope-free media, multiply infected as before, and the purified phage partitioned and the radioactivity measured. As shown in Table I, the specific activity of the isolated phage DNA P was about 25 per cent that of the bacteria in Experiment III and 17 per cent in Experiment IV, indicating that this portion of the virus P was derived from the labeled host. The results are in good agreement with the complementary experiment with the labeled lactate medium and unlabeled cells (Experiment I, Table I).

Differentially Labeled Cells As Source of Phage P—The acid-soluble P fraction of the bacteria had been suggested by Cohen (5) as the most likely source of the P contributed by the bacterium to the virus. This possibility was tested by techniques suggested by the work of Spiegelman and Kamen (13). The latter workers obtained yeast differentially labeled with P^{32} in the several P fractions by allowing uniformly labeled yeast cells to metabolize glucose anaerobically in an isotope-free N-free medium. In yeast the specific activity of the acid-soluble fraction (which was rapidly turning over its P) was greatly diminished, while the radioactivity of the nucleic acids was only slightly lowered. Upon application of this principle to *E. coli* it was found that aerobic oxidation of lactate by P^{32} -labeled bacteria in isotope-free N-free medium resulted in differentially labeled cells. As before, the specific activity of the original labeled bacteria and of each of the several P fractions approximated that of the medium (average value of about 480 counts per minute per microgram of P for the fractions compared to 506 counts per minute per microgram of P for the medium). However, after incubation with aeration for 5 hours in lactate in the absence of a nitrogen source, the specific activity of the acid-soluble P was 195 counts per minute per microgram of P compared to 415 for DNA or 430 for RNA.² The cells were again centrifuged and washed and then infected with phage in isotope-free medium.³

² The fall in radioactivity of the acid-soluble P is greatest in the first hour of incubation. This is probably due to the rapid equilibration of bacterial inorganic

Although the relative radioactivity of the acid-soluble P of the bacteria (compared to that of the total bacteria) was diminished by about one-half in this experiment, the relative radioactivity of phage DNA to total bacterial P was elevated to 37 per cent, as compared to 17 to 24 per cent in previous experiments. The relative radioactivity of phage DNA to bacterial DNA was, if anything, increased, compared to that obtained earlier (31 per cent as compared to 26 per cent found in Experiment III, and 17 per cent in Experiment IV). The fact that a gross change in the specific radioactivity of bacterial acid-soluble phosphorus is not paralleled by a similar change in the radioactivity of the virus progeny suggests that the intracellular metabolic pool of low molecular P is not the major source of bacterial P appearing in the liberated virus.

In a number of instances it was possible to calculate whether any single P fraction of the bacteria contained sufficient radioactivity and P to account for both the radioactivity and P in the DNA of virus liberated from a single host cell. The experimental quantities used in this calculation were (1) the specific radioactivity (counts per minute per microgram of P) of each P fraction of a single bacterium (a figure determined by the partition analysis of a known number of cells obtained from the bacterial suspension just prior to infection), (2) the yield of phage per bacterial cell, (3) the P and DNA content per phage, and (4) the specific radioactivity of phage DNA P. The results of these calculations, contained in Table II, provide some information as to the nature of the bacterial precursor of virus P.

Three points are made apparent by comparison of the DNA content and radioactivity of the virus liberated per cell with that of the individual P fractions of a single bacterium: (1) The DNA content of the virus liberated per cell (Table II, Line 9) is from 1 to 2 times as great as the original DNA content of the host organism (Line 3). It is to be noted that the synthesis of DNA stimulated by infection depends upon the yield of virus, and in some experiments larger increases than those reported in Table II have been observed. (2) Since the phosphoprotein fraction of the bacteria (Line 5) contains insufficient P or radioactivity to account for that found in the isolated phage, this fraction can be excluded as the sole or major bacterial precursor of phage P. Acid-soluble P, alcohol-soluble P, DNA, and RNA are all individually adequate to provide the bacterial contribu-

phosphate (about 50 per cent of bacterial acid-soluble P) with the P of the medium. Thereafter, the radioactivity of the acid soluble fraction falls more slowly (5 to 10 per cent per hour) and is still falling at the end of 7 hours.

³ The yield of phage liberated per bacterial cell after multiple infection was diminished in this experiment, perhaps because of the treatment incurred in differential labeling.

tion to phage P as well as the radioactivity of the phage. For example, in Experiment III (see Tables I and II) the total phage P derived from a single bacterial cell is equivalent to 40 per cent of the DNA P of the host cell ($(100 \times 0.26 \times 3.4 \times 10^{-9} \gamma \text{ of P}) / 2.2 \times 10^{-9} \gamma \text{ of DNA P}$), and the radioactivity of the virus liberated is 40 per cent that contained in the DNA of a single organism. (3) Similar comparison in Experiments III

TABLE II

Average P Content and Radioactivity of Single Bacterial Cell in Relation to Yield of Virus per Cell

Phosphorus fraction of <i>E. coli</i>	Experiment III Labeled cells in unlabeled medium		Experiment V Differentially labeled cells		Experiment VI Labeled cells in labeled medium	
	P content	Radioac- tivity	P content	Radioac- tivity	P content	Radioac- tivity
A. P content and radioactivity of P fractions per single bacterial cell prior to infection						
	$\gamma \text{ P} \times 10^{-3}$	c.p.m. $\times 10^{-7}$	$\gamma \text{ P} \times 10^{-3}$	c.p.m. $\times 10^{-7}$	$\gamma \text{ P} \times 10^{-3}$	c.p.m. $\times 10^{-7}$
1. Acid-soluble.	2.8	2.25	2.6	5.0	4.25	26.3
2. Alcohol-soluble.	1.7	1.3	1.7	5.8	2.0	9.7
3. DNA.	2.2	1.66	2.7	11.2	2.6	11.8
4. RNA.	4.9	3.85	4.1	17.6	10.2	52.5
5. Phosphoprotein.	0.55	0.38	0.7	0.4	0.48	2.0
6. Total bacterial P.	12.15	9.44	11.8	40.0	19.53	102.3
B. P content and radioactivity of phage liberated per bacterial cell						
7. Yield of phage per bacterium*.	80		53		140	
8. $\gamma \text{ P}$ per phage ($\times 10^{-11}$)	4.5		4.5		4.0	
9. Total $\gamma \text{ DNA phage P} \dagger$ ($\times 10^{-9}$)	3.4		2.3		5.3	
10. Total radioactivity of phage DNA P (10^{-7} c.p.m.)	0.67		3.0		42	

The roman numerals refer to experiment numbers of Table I and Table III (see later).

* (Titer of virus in lysate)/(titer of bacteria at time of infection).

† Assuming DNA phage P to total phage P = 0.95 (2).

and V of the possible degree of conversion of acid-soluble P to phage P was made. Inspection of the data (Lines 1 and 10) reveals that, when the relative radioactivity of acid-soluble P was halved by differential labeling (Experiment V), the ratio of total radioactivity of phage progeny to that of bacterial acid-soluble P was doubled (60 per cent in Experiment V compared to 30 per cent in Experiment III). This result suggests either that (a) there is a highly efficient utilization of heterogeneous low molecular

weight P intermediates in the synthesis of virus P, or (b) a P fraction other than the acid-soluble one is the major specific source of the bacterial contribution to phage P.

The relation of yield of virus per cell to the amount of bacterial P contributed is shown in Table III. It can be seen that, although the yield of virus varies as much as $3\frac{1}{2}$ -fold, there is no corresponding wide variation in the bacterial P contribution. This lack of correlation would appear to support the view that there is a specific substance which is transferred from host to virus.

Organic P of Nutrient Broth As Source of Bacterial P and Phage P—In previously reported studies of the origin of virus P in the *E. coli* bacteriophage system (3, 5), only synthetic lactate medium containing inorganic phosphate was used as the external source of phage P. Since nutrient broth (which contains varying quantities of organic P) is known to have a stimulating effect on the yield of T₆ bacteriophage per bacterium (1), it

TABLE III
Yield of Virus per Host Cell Compared to Contribution of Bacterial P

Experiment No.	Yield of phage* per bacterium	Per cent of virus P derived from bacterial host
I	180	24
II	128	21
III	80	26
IV	160	17
V	53	31

* (Titer of virus in lysate)/(titer of bacteria at time of infection).

was thought of interest to investigate the origin of phage P in this more complex medium. In addition, in nutrient broth but not in synthetic medium, T₆ phage can also be grown by the single infection procedure which yields many generations of virus, as well as by the multiple infection procedure previously employed (see (1)). Although the technique of single infection does not lend itself to the labeling of separate components in the growth system, as does the procedure of multiple infection, it can provide some information on the precursors of phage P under conditions different from those used in the previous experiments.

Preliminary experiments involving incubation of normal, *i.e.* uninfected, *E. coli* in nutrient broth labeled with NaH₂P³²O₄ demonstrated that the bacteria selectively utilize the organic P constituents of broth for growth. Experiments 1, 2, and 3 in Table IV show that the specific activity of bacteria grown in broth is significantly lower than that of the medium inorganic P (or of the total medium P), whereas in synthetic medium all the P

fractions of the bacterium had the same specific activity as the medium. In all experiments DNA P had the lowest specific activity and acid-soluble P the highest activity of all the bacterial fractions studied. This disparity was interpreted as indicating that the DNA was synthesized by preferential assimilation of unlabeled organic P precursors in broth rather than from inorganic P. Since the amount of inoculating bacteria was equal to only 1 to 2 per cent of the yield, the lower specific radioactivity of the bacterial DNA cannot be ascribed to retention of the DNA P of the original bacteria.

When the bacteria were first grown in labeled nutrient broth and then infected *multiply* with T₆ phage in the same labeled medium, the relative

TABLE IV

Growth of Uninfected E. coli and of Bacteriophage T₆ on Labeled E. coli in Nutrient Broth Containing Radioactive Phosphorus

Experiment No.	Specific radioactivity, c.p.m. per γ P					Relative radioactivity†	
	Me- dium*	Bacteria				Bacter- ial DNA Medium	Virus DNA Medium
		Total P	Acid- soluble P	Total nucleic acids	DNA		
						per cent	per cent
1. Uninfected bacteria.....	753	505	622	500	455	60.5	
2. " "	4130	2710	3250	2750	2300	55.7	
3. " "	123	86	89	86	68	55	
VI. Multiple infection.....	785						91
VII. " "	4080						94.5
VIII. Single "	114						51

* Expressed as counts per minute per microgram of inorganic P.

† Ratio of specific activities $\times 100$.

radioactivity of the DNA of the isolated phage was 91 to 95 per cent that of the medium (Experiments VI and VII, Table IV). This is to be contrasted with the values of 55 to 60 per cent obtained for bacterial DNA. On the other hand, when a bacterial subinoculum growing in labeled broth was *singly* infected with T₆ prior to the inoculation of the bulk of the labeled broth, the relative radioactivity of the DNA of the virus produced was only 50 per cent that of the medium (Experiment VIII, Table IV). This value for the relative radioactivity, repeatedly obtained (14), is markedly different from that for phage grown by the multiple infection techniques but quite close to the values obtained for bacterial DNA.

The most plausible interpretation of these experiments, and likewise

that most amenable to test, was that the larger amounts of labeled inorganic P in phage DNA during multiple infection were to be attributed to depletion of the medium of an organic P compound preferentially assimilated during growth of the bacteria. The experiments described in Table V support this hypothesis.

Depletion of Broth Medium of Organic P Precursors of Bacterial DNA—Evidence for the depletion of broth medium of organic P precursors of bacterial DNA was sought in the following way: 250 ml. of broth containing 5 μ c. of P³² per ml. were inoculated with *E. coli* and the bacteria grown to the concentration normally used for multiple infection, *i.e.* 2.5×10^8 cells per ml. One aliquot of this culture (Experiment A, Table V) was removed and the cells centrifuged, washed, and fractionated. The rest of

TABLE V
*Specific Radioactivity of Phosphorus Fractions of E. coli Grown to Different Concentrations in Broth Containing P³²**

Final bacterial concentration (cells per ml.)	Experiment A		Experiment B		Experiment C	
	<i>c.p.m.</i> per γ P	<i>per cent</i> †	<i>c.p.m.</i> per γ P	<i>per cent</i> †	<i>c.p.m.</i> per γ P	<i>per cent</i> †
Media inorganic P.....	2590	100	2730	100	2460	100
Total media P.....	2300	89	2480	91	2460	100
Acid-soluble P.....	2160	83.5	2450	80	2630	106
Alcohol-soluble P.....	1640	63	2250	83	2060	84
RNA + protein P.....	1850	71	2310	85	2710	110
DNA P.....	1540	59.5	2390	88	2130	87

* For a description of the experiments, see the text.
† Specific activity of fraction/specific activity of medium $\times 100$.

the original culture (Experiment B) as well as the supernatant from the aliquot centrifuged (Experiment C) was again incubated until the amount of bacterial DNA synthesized in both cases approximated the 2- to 3-fold increase in DNA due to phage reproduction which would have taken place upon infection; that is, the samples were grown respectively to 8.5×10^8 cells per ml. for the original culture (Experiment B, Table V), and 4×10^8 cells per ml. for the depleted supernatant broth (Experiment C, Table V). The specific activities of the whole media and of the P fractions of the bacteria are given in Table V. It can be seen that the specific activity of the DNA of cells from fresh media (Experiment A) is 60 per cent that of the medium, but that the specific activity of DNA of cells grown to a higher titer or in depleted broth is much higher (88 per cent) and is similar to that of the corresponding medium.

DISCUSSION

It has been known for some time that bacteriophage-infected cells require an exogenous source of nitrogen and oxidizable carbon for the synthesis of virus (15), and recently it has been reported that the absence of phosphorus in the medium severely limits the yield of virus (16). Similarly, one step growth experiments with T_2 bacteriophage (16) and T_6 bacteriophage (1) have demonstrated that the yield of virus is increased by various amino acids, nucleosides, or vitamins. Likewise, Price (17) claims that multiplication of staphylococcus phage requires a substance present in yeast extract which is not necessary for the growth of the host cell. Such experiments on the multiple nutritional requirements of bacteriophage have suggested the need for revision of earlier theories of virus reproduction which proposed an autocatalytic conversion of cellular protein into virus protein (18, 19). Direct evidence for the precursors of virus nucleic acid and protein is best obtained by isotopic studies. The experiments with P^{32} described in this paper provide evidence on the nature of a bacterial precursor of virus P but show that quantitatively only a small fraction of the total bacterial P is utilized in virus synthesis.

The results given in Tables I and III, and the calculations, summarized in Table II, reveal that the *absolute* amount of bacterial P utilized in bacteriophage synthesis depends upon the yield of virus per cell, but that the proportion of phage P derived from the host is relatively constant, comprising from 17 to 30 per cent of total phage P. Quantitatively, only about one-twelfth of the total bacterial P was converted to phage P in the experiments described. On the other hand, about 70 to 80 per cent of the virus P was derived from the inorganic phosphate of the medium. The latter result is in accord with previous observations of Cohen (5) made from radiophosphorus studies on the growth of bacteriophages T_2 and T_4 in synthetic medium. Similarly, in both studies a net synthesis of DNA was observed upon infection of the bacterial cell. These experiments thus demonstrate that only a small fraction of bacterial protoplasm is utilized in virus synthesis. This conclusion is contrary to that reached by Wyckoff (20, 21), who inferred from electron micrographs that bacterial protoplasm is completely converted to bacteriophage after invasion of the cell by the virus.⁴

An important clue to virus reproduction would seem to lie in the nature of the material contributed by the bacterium to the virus and in the mech-

⁴ It should be pointed out that the N to P ratio of the host organism is greater than that of the virus (2, 22). Accordingly, the utilization of bacterial N for the synthesis of bacteriophage would have to proceed at greater efficiency than that for P if bacterial N is incorporated into phage to the same degree as bacterial P.

anism of transfer. Cohen (5) has suggested that some of the P of the host may be incorporated into bacteriophage by way of "the intracellular pool of inorganic P or low molecular weight organic P which can equilibrate with P assimilated after infection." This hypothesis seemed most plausible, but two lines of evidence indicate that some other bacterial fraction is the precursor of virus P: (a) The experiment performed with differentially labeled bacteria showed that there was no direct relation between the radioactivity of the acid-soluble fraction of the host and the radioactivity found in the virus produced; and (b) the proportion of phage P derived from the host was not greatly affected by the yield of virus per cell (Table III). If, as is commonly supposed, the virus is synthesized at either a linear or a logarithmic rate, the first virus particles formed should have a higher proportion of the labile P of the intracellular pool, and thus the percentage of host P appearing in the virus would be dependent on the time of the experiment or the yield per cell.

The results cited above suggest that the bacterium contains a stable and possibly specific precursor of a moiety of the virus P. Our experiments have indicated that acid-soluble P is not that source, and have also excluded protein P because of the insufficiency of both P and radioactivity in that fraction. Bacterial phospholipide P can be discounted as the source, for Cohen (4, 5) has demonstrated that phospholipides do not turn over in bacteriophage-infected cells. Similarly, RNA does not appear to be the source of bacterial contribution to virus P, for evidence exists to show that RNA is not a significant precursor of DNA in the *E. coli* bacteriophage system (4, 5). In view of the above evidence, excluding or minimizing the participation of other bacterial P fractions in the synthesis of phage DNA, it seemed warranted for to us to suggest (3) that bacterial desoxyribonucleic acid is the major source of the bacterial contribution to phage P. The implications of such a transfer, possibly mediated by way of nucleotides rather than via intact DNA, are considerable. However, speculation as to the significance of such transfer will be withheld for a later communication which will present the results of studies on utilization of bacterial N and bacterial purines for the synthesis of phage nucleoprotein.

The experiments performed by growth of normal or infected labeled cells in labeled broth are simply explained by the assumption that broth contains an organic P compound (adenylic acid?) preferentially utilized for synthesis of DNA. This hypothesis is compatible with the known stimulating effect on phage growth exhibited by small additions of broth made to synthetic medium in one-step growth curve experiments (1). When the supply of the organic P precursor is exhausted by growth of bacteria to high concentrations, the cells must draw upon the labeled

inorganic P of the medium for synthesis of DNA. However, when phage is formed before depletion of the medium, as occurs in the single infection growth technique, the medium precursors used preferentially are the same unknown organic P compounds of broth. Thus, no difference in the synthesis of virus during single *versus* multiple infection need be postulated, and it appears probable that the same broth precursors and probably the same enzymic mechanisms are used for synthesis of both bacterial and phage DNA.

SUMMARY

The origin of virus phosphorus in the *E. coli* T₆ bacteriophage system has been investigated with P³²-labeled host cells or medium. In synthetic (lactate) medium, the inorganic phosphate of the medium is the ultimate source of 70 to 80 per cent of the bacteriophage phosphorus; the remainder of the phage P is derived from host material originally present at the time of infection. In nutrient broth the proportions of phage P arising from the medium and the host are similar to those found for synthetic medium, but broth also contains an unidentified phosphorylated organic precursor of desoxyribonucleic acid for both the virus and its host. The nature of the bacterial precursor of phage nucleic acid P was investigated by comparison of the radioactivity and P content of the virus liberated from a single host cell with that of the individual phosphorylated fractions of the cell and also by use of differentially labeled bacteria. The results show that only a small fraction of total bacterial protoplasm is converted to bacteriophage and indicate that the desoxyribonucleic acid of the host organism is the major specific source of the bacterial contribution to virus phosphorus.

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BIBLIOGRAPHY

1. Putnam, F. W., Kozloff, L. M., and Neil, J. C., *J. Biol. Chem.*, **179**, 303 (1949).
2. Kozloff, L. M., and Putnam, F. W., *J. Biol. Chem.*, **181**, 207 (1949).
3. Putnam, F. W., and Kozloff, L. M., *Science*, **108**, 386 (1948).
4. Cohen, S. S., *J. Biol. Chem.*, **174**, 281 (1948).
5. Cohen, S. S., *J. Biol. Chem.*, **174**, 295 (1948).
6. Reid, A. F., in Wilson, D. W., Nier, A. O. C., and Reimann, S. P., Preparation and measurement of isotopic tracers, Ann Arbor (1946).
7. Schmidt, C. F., *J. Bact.*, **55**, 705 (1948).
8. Kozloff, L. M., and Putnam, F. W., *Federation Proc.*, **8**, 215 (1949).
9. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **161**, 83 (1945).
10. Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945).
11. Born, H. S., Lang, A., and Schramm, G., *Arch. ges. Virusforsch.*, **2**, 461 (1943).

12. Doermann, A. H., *J. Bact.*, **55**, 257 (1948).
13. Spiegelman, S., and Kamen, M., *Cold Spring Harbor Symposia Quant. Biol.*, **12**, 211 (1947).
14. Putnam, F. W., Kozloff, L. M., and Evans, E. A., Jr., *Federation Proc.*, **7**, 179 (1948).
15. Spizizen, J., *J. Infect. Dis.*, **73**, 212, 222 (1943).
16. Fowler, C. B., and Cohen, S. S., *J. Exp. Med.*, **87**, 259 (1948).
17. Price, W. H., *J. Gen. Physiol.*, **32**, 301 (1949).
18. Krueger, A. P., and Scribner, E. J., *J. Gen. Physiol.*, **22**, 699 (1938-39).
19. Bordet, J., *Proc. Roy. Soc. London, Series B*, **107**, 398 (1931).
20. Wyckoff, R. W. G., *Proc. Soc. Exp. Biol. and Med.*, **66**, 42 (1947).
21. Edwards, O. F., and Wyckoff, R. W. G., *Proc. Soc. Exp. Biol. and Med.*, **64**, 16 (1947).
22. Taylor, A. R., *J. Biol. Chem.*, **165**, 271 (1946).

BIOCHEMICAL STUDIES OF VIRUS REPRODUCTION

IV. THE FATE OF THE INFECTING VIRUS PARTICLE*

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Despite frequent speculation concerning the mode of action of viruses, few biochemical studies of the virus-host cell relationship have been undertaken, and the sequence of steps in virus multiplication remains largely unknown. Any tenable theory of virus reproduction must consider both the rôle and the fate of the original infecting virus particle, and, at present, little evidence is available on this subject. Though the isotopic tracer technique lends itself to a study of this problem through the use of labeled virus, only one early and inconclusive experiment of this nature has been reported (2). In the course of study of virus precursors in the *Escherichia coli* T₆ bacteriophage system (3-6), procedures were developed for the preparation of P³²-labeled bacteriophage which made tracer experiments feasible under conditions of controlled infection. The research described below reveals that, while in the course of bacteriophage multiplication a significant amount of the phosphorus of the infecting virus particle appears in the progeny, much of the P is liberated into the medium in a soluble form. The isotopic data, together with observations in bacteriophage genetics (7-10), indicate that the original infecting virus particle does not survive reproduction.

EXPERIMENTAL

Procedure

Preparation of P³²-Labeled Bacteriophage—T₆r⁺ bacteriophage was labeled by growth on *E. coli* in broth containing 2 to 4 μ c. of P³² per ml. After purification (4, 5) the radioactive phage was used for infection of bacteria actively growing in nutrient broth. The progeny of the labeled infecting phage was isolated as related below, and the radioactivity was measured in all the solutions obtained in the course of purification. The conditions for growth of the phage and the methods of assay and of measuring radioactivity have been previously described (4-6). About 95 per cent of the P found in the purified phage is present as desoxyribonucleic acid (DNA) (5). Preparations of bacteriophage obtained by these meth-

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ods have been shown to be essentially homogeneous in the ultracentrifuge and in the electrophoresis apparatus (4, 5). The labeled virus used for inoculation possessed somewhat lower infectivity (gm. of N per plaque-forming particle) than that customarily obtained. This was apparently due to the radioactivity incorporated into the phage. However, prior to infection 98 per cent of the radioactivity of the purified virus concentrate was sedimentable with the virus at $20,000 \times g$, and, upon infection, the adsorption of radioactivity by the bacteria paralleled the adsorption of the phage. Previous experiments had demonstrated that not more than 0.1 per cent of the DNA phosphorus of the phage exchanges with the inorganic phosphate of the medium (6).

Infection with Labeled Virus—Bacteria, actively growing in isotope-free broth at a concentration of about 2×10^8 cells per ml., were infected with labeled virus at a phage to bacterium ratio of about 3:1. Under these conditions bacterial multiplication ceases (4, 11, 12), lysis inhibition ensues (11), and the phage produced represents for the most part a single generation of virus. In several experiments the bacteria were first singly infected with labeled virus (one phage per bacterium) and additional unlabeled virus was added at a later time.

Isolation of Phage Progeny—The isolation of the newly formed virus was accomplished by one of two methods: (1) In the first experiments described, the infected bacteria were separated from unadsorbed phage 15 minutes after infection by 10 minutes centrifugation at $4000 \times g$. The bacteria were resuspended in sterile broth and incubated with aeration at 37° overnight. The virus liberated was purified by repeated differential centrifugation in the high speed angle centrifuge, one step at $2000 \times g$ for 15 minutes to remove bacterial detritus and unlyzed bacteria, another at $20,000 \times g$ for 2 hours to sediment the phage. In this method the radioactivity was measured in the crude lysate, in the supernatants obtained at low and high speed, and in the phage concentrate. (2) In other experiments in which larger volumes of culture were employed the unadsorbed phage was not removed and the newly formed phage was purified as previously described (4); *i.e.*, by preliminary filtration through a Mandler candle, followed by concentration in the Sharples supercentrifuge and then differential centrifugation as above. The two procedures yielded similar products. The low speed supernatant solution in the first method is comparable to the filtered lysate in the second, and the high speed supernatant solution is analogous to the Sharples effluent.

Results

Multiple Infection with Labeled Phage—The distribution of radioactivity in the solutions obtained after multiple infection of *E. coli* with P^{32} -labeled

bacteriophage T_6 and purification of the progeny is shown in Table I. In this experiment 800 ml. of broth containing actively growing bacteria at a concentration of 2.5×10^8 cells per ml. were inoculated with 2 ml. of radioactive phage having a titer of 3.1×10^{11} particles per ml. and a radioactivity of 50,000 counts per minute per ml. 15 minutes after infection the unadsorbed phage was removed by centrifugation. At this time 60 per cent of the phage and 67 per cent of the radioactivity had been adsorbed by the bacteria. The titer of the final lysate was 5.6×10^{10} phage per ml. About 25 ml. of phage having a titer of 8.5×10^{11} particles per ml. and an infectivity of $10^{-15.87}$ gm. of N per plaque were obtained on purification by differential centrifugation in the angle centrifuge. The radioactivity of the phage was calculated by multiplying the radioactivity per purified virus particle by the titer of the lysate. Practically all of the radioactivity of the recovered phage were in the nucleic acid fraction. 40

TABLE I

Distribution of Radioactivity after Multiple Infection of E. coli with P^{32} -Labeled Bacteriophage T_6

Material	Titer	Radioactivity		
	phage per ml. $\times 10^1$	c.p.m. per ml.	per cent	c.p.m. per phage $\times 10^{-3}$
Lysate.....	5.8	84.6	100	1.4
Low speed supernatant.....	6.2	76	89.8	1.2
High " ".....	0.027	41.5	49.2	150
Phage concentrate.....	85	33.1* (485.2)	39.1	0.57
Purified phage concentrate.....	120	34.5* (718)	40.8	0.59

* Corrected to original volume; actual values given in parentheses.

per cent of the added radioactivity appeared in the phage progeny, and this value was unchanged after a second cycle of centrifugation. Half of the total radioactivity was found in the high speed supernatant solution. This solution was essentially free of phage and the radioactivity per phage particle in this supernatant solution was about 300 times as great as in the purified phage concentrate.

Mixed Infection with Labeled and Unlabeled Phage—In a second experiment (Table II), the bacteria were first infected with labeled phage at a phage to bacterium ratio of 1. 3 minutes after infection, 68 per cent of the phage and 67 per cent of the added radioactivity had been adsorbed. At this time unlabeled phage was added to the infected bacterial suspension, so that there were two additional phage particles per bacterium. The purpose of the experiment was to ascertain whether the first phage particle adsorbed contributed more radioactivity to the progeny than did

virus adsorbed at a later time.¹ However, the same proportion of the radioactivity was found in the purified virus progeny as previously.

Growth of Phage without Removal of Unadsorbed Labeled Phage—Another series of experiments performed without removal of the unadsorbed phage, and by using the supercentrifuge for preliminary concentration of the phage, gave essentially similar results. The data are summarized in Table III. Despite the lack of removal of unadsorbed phage, the percentage of the added radioactivity that appeared in the newly formed virus was similar but slightly lower than in the previous experiments. As before, no difference in the result was found whether each bacterium was infected simultaneously with several labeled virus particles (multiple infection, Experiments I to III, Table III) or whether it was infected first

TABLE II
Distribution of Radioactivity after Mixed Infection of E. coli, First with P³²-Labeled Bacteriophage T₆ and Then with Unlabeled Bacteriophage

Material	Titer	Radioactivity		
	<i>phage per ml. $\times 10^{10}$</i>	<i>c.p.m. per ml.</i>	<i>per cent</i>	<i>c.p.m. per phage $\times 10^{-10}$</i>
Lysate.....	6.6	38.2	100	5.8
Low speed supernatant.....	5.8	32.4	85	5.6
High “ “.....	0.041	13.2	35	320
Phage concentrate.....	105	16.0* (257.6)	42	2.4

* Corrected to original volume; actual values given in parentheses.

with one labeled particle, and 3 minutes later unlabeled phage was added to the culture (Experiment IV).

Nature of Unsedimentable Phosphorus—In Experiment III described above it was found that 45 per cent of the P³² in the Sharples effluent could not be precipitated by cold 5 per cent trichloroacetic acid and, hence,

¹ When large numbers of phage are used to infect large numbers of bacteria at a given multiplicity (ratio of phage to bacteria), the virus content is distributed among the bacteria in different multiplicities. The actual spread of the multiplicities is a Poisson distribution. Thus it can be calculated that, in the experiment in which bacteria were infected first with one labeled phage and then with two unlabeled phages, 64 per cent of the labeled phages were the first virus particles to be adsorbed by bacteria (the other labeled particles being the second or third phages adsorbed by multiply infected bacteria). However, in the experiment in which the ratio of labeled phage to bacteria was 3, the fraction of the total radioactivity contained in all the first particles adsorbed by each bacterium was 32 per cent. Accordingly, if only the first phage adsorbed contributed its P to the progeny, the radioactivity of the liberated virus should have been twice as great in the first experiment above as in the second.

was apparently not nucleic acid or attached to protein. Half of the radioactivity of the effluent which was soluble in cold trichloroacetic acid was precipitable as inorganic phosphate by alkaline magnesia mixture. The results indicate that much of the nucleic acid P of the original infecting virus particle was now present in the medium in a low molecular weight form. The same conclusion is obtained by inspection of the figures for total radioactivity per phage particle found in the purified concentrate compared to that in the Sharples effluent (see Table I, the last column).

TABLE III

Distribution of Radioactivity after Infection of E. coli with P³²-Labeled Bacteriophage T₆

Experiment No.	Crude lysate	Filter candle	Sharples effluent	Phage progeny
% radioactivity				
I*	100	15	63	22
II*	100	9	60	31
III*	100	11	57	32
IV†	100	0	70	31
C.p.m. per phage × 10 ³				
I*	1.9		140	0.4
II*	5.7		760	1.9
III*	0.59		18	0.19
IV†	1.4		230	0.44

* Multiple infection.

† Single infection with labeled phage, secondary infection with unlabeled phage.

DISCUSSION

Although all hypotheses of virus reproduction assume some sort of autocatalytic reaction (13), it cannot yet be said with certainty whether viruses multiply by logarithmic replication, by successive linear duplication, or otherwise. It seems probable, however, that, for all viruses so far studied, a single particle is sufficient to initiate an infection under ideal conditions of susceptibility of the host (14, 15). Indeed, for the coliphages (16) each active phage particle will give one plaque (phage colony). The original particle disappears within the cell and after reproduction cannot be distinguished from its progeny. In the first isotopic experiments on the fate of the infecting virus particle, Stanley (2), using P³²-labeled tobacco mosaic virus, found that most of the radioactivity finally appeared in normal components of the plant cell and only a small percentage appeared in the newly isolated virus. He assumed that but a small fraction of the

inoculating virus entered the cell as intact infectious virus and concluded that the incorporation of P^{32} into new virus from active virus could not be distinguished from incorporation from virus disintegration products. This quandary is resolved in our experiments by the removal of unadsorbed phage. The *E. coli* bacteriophage system, moreover, possesses the advantage that multiple infection of the bacteria with phage of the wild type (r^+) strain produces prolonged inhibition of lysis and gives large yields of virus comprising, for the most part, a single generation (4, 11, 12).

In all the experiments consisting of multiple infection of bacteria with labeled phage, the bulk of the radioactivity (49 to 63 per cent) was found in the medium as unsedimentable low molecular weight P. These results indicate that disintegration of the infecting virus particles occurs. The latter conclusion is corroborated by the finding that the radioactivity per phage particle in the supernatant solutions was at least 100-fold greater than that in the purified virus concentrate. About 10 to 15 per cent of the isotope was associated with filterable bacterial debris,² and a significant portion (22 to 42 per cent) appeared in the isolated virus.

Although it could not be determined by direct experiment whether the radioactivity in the liberated phage was contained in unaltered original virus particles or was distributed among the progeny, the weight of the evidence favors the latter hypothesis. Hershey (8) has demonstrated that more than one particle of the same phage type can participate in reproduction inside the same bacterial cell.³ Delbrück and Bailey (17) have shown that, when a single bacterium is infected with two coliphages of closely related strains, both phages are adsorbed, and each multiplies successfully. Our own experiments indicate that, when bacteria are infected, first with one labeled particle and 3 minutes later with two unlabeled phages, the same fraction of the radioactivity appears in the isolated virus as when bacteria are simultaneously infected with several labeled phage particles. These data demonstrate (1) that several virus particles of the same or closely related strains may reproduce in a single cell without interference but not without interaction (see below), and (2) that the first particle adsorbed does not exert an inhibiting effect on other particles when the primary infection precedes secondary infection by only 3 minutes. Since each adsorption is equally fruitful, it seems probable that each multiplying virus meets the same fate; namely, disintegration during the

² Not all the bacteria lyse simultaneously. It has been suggested that the first phages liberated are secondarily adsorbed on as yet unlysed cells, causing delayed lysis (11). On rupture of the latter bacteria, the newly formed but secondarily adsorbed phage may remain attached to the bacterial debris, accounting for the fraction of the radioactivity usually found in that material (10 to 15 per cent).

³ Dulbecco (10) presents evidence that the maximum number of particles of bacteriophage that can participate in intracellular growth in a single cell is 8 to 10.

process of reproduction. Further support for the view that the original virus particle is disrupted is found in the one-step growth curve studies of Doermann (18). By inducing premature lysis of infected bacteria through the use of sonic vibration or chemical agents, Doermann found that the average intracellular virus content is far less than 1 in the first half of the latent period (12).

The most plausible interpretation of our data is that all infecting virus particles are destroyed during reproduction, each contributing essential phosphorylated material to the progeny. Using the idea of recombination (17), Luria and Dulbecco (7, 9) have suggested from studies of coliphage inactivated by ultraviolet light that a transfer of "self-reproducing units" may take place, resulting in reactivation of the inactive particles. The ultraviolet light-absorbing loci involved in this interaction between two individually non-infective particles number 30 to 50 and have been compared to genes, and the virus particle itself to a "gene complex." Moreover, Hershey and Rotman (8, 19) have suggested that T_2 , T_4 , and T_6 coliphages are capable of mutual transfer of hereditary characteristics such as plaque type, host range, etc., when adsorbed in the same host cell. For example, if a single cell is infected with two virus particles bearing different genetic markers, each phage reproduces, but all possible "recombinants" are likewise obtained. Both workers, from two different lines of evidence, have arrived at the idea of independently multiplying subunits. Indeed, Luria (7) has suggested that reproduction takes place by independent reproduction of a number of units and incorporation of these into the final phage particles. Further evidence for the existence of "immature" phage particles is summarized by Cohen (12).

The P^{32} found in the isolated virus may be combined in one of the genetic units postulated by Hershey and Rotman (8, 19) and Luria and Dulbecco (7, 9). If this assumption is correct, these units must contain about one-third of the phosphorus of the phage; for this is the fraction appearing in the progeny. Finally, it should be pointed out that quantitatively the infecting virus contributes only a minute portion of the P of the progeny.

SUMMARY

The fate of the infecting virus particle has been investigated with P^{32} -labeled *Escherichia coli* bacteriophage T_6 . The labeled virus used for infection was prepared by growth on *E. coli* in broth containing radio-phosphorus and was purified by differential centrifugation. Bacteria in isotope-free broth were infected with the purified radioactive phage under conditions producing largely a single generation of virus. The greater part of the isotope was found in the medium as unsedimentable low molecular P, but a significant portion appeared in the liberated virus. The

fraction of the radioactivity appearing in the liberated virus was the same, whether bacteria were multiply infected simultaneously with several labeled phage particles or mixedly infected successively with labeled and unlabeled bacteriophage. The results indicate that the original infecting particle is disrupted within the cell and that about one-third of its phosphorus is transferred to the progeny. The significance of this conclusion is discussed in relation to the hypothesis that bacteriophage reproduction takes place by independent reproduction of a number of subunits and incorporation of these into the final phage particles.

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BIBLIOGRAPHY

1. Kozloff, L. M., and Putnam, F. W., *Federation Proc.*, **8**, 215 (1949).
2. Stanley, W. M., *J. Gen. Physiol.*, **25**, 881 (1941-42).
3. Putnam, F. W., and Kozloff, L. M., *Science*, **108**, 386 (1948).
4. Putnam, F. W., Kozloff, L. M., and Neil, J. C., *J. Biol. Chem.*, **179**, 303 (1949).
5. Kozloff, L. M., and Putnam, F. W., *J. Biol. Chem.*, **181**, 207 (1949).
6. Kozloff, L. M., and Putnam, F. W., *J. Biol. Chem.*, **182**, 229 (1950).
7. Luria, S. E., *Proc. Nat. Acad. Sc.*, **33**, 253 (1947).
8. Hershey, A. D., *Genetics*, **31**, 620 (1946).
9. Luria, S. E., and Dulbecco, R., *Genetics*, **34**, 93 (1949).
10. Dulbecco, R., *Genetics*, **34**, 126 (1949).
11. Doermann, A. H., *J. Bact.*, **55**, 257 (1948).
12. Cohen, S. S., *Bact. Rev.*, **13**, 1 (1949).
13. Northrop, J. H., Kunitz, M., and Herriott, R. M., *Crystalline enzymes*, New York, 2nd edition, 239 (1948).
14. Lauffer, M. A., and Price, W. C., *Arch. Biochem.*, **8**, 449 (1945).
15. Beard, J. W., *J. Immunol.*, **58**, 49 (1948).
16. Ellis, E. L., and Delbrück, M., *J. Gen. Physiol.*, **22**, 365 (1938-39).
17. Delbrück, M., and Bailey, W. T., Jr., *Cold Spring Harbor Symposia Quant. Biol.*, **11**, 33 (1946).
18. Doermann, A. H., *Carnegie Inst. Washington Yr. Bk.*, **47**, 176 (1947-48).
19. Hershey, A. D., and Rotman, R., *Genetics*, **34**, 44 (1949).

A COMPARISON OF HUMAN γ -GLOBULINS IN THEIR REACTIVITY WITH RABBIT ANTI- γ -GLOBULIN BY THE QUANTITATIVE PRECIPITIN METHOD*

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Because of their specificity and sensitivity, quantitative immunochemical methods have proved among the most useful for the estimation of small quantities of certain proteins and polysaccharides, even in the presence of large quantities of other substances ((1-7), cf. (8, 9)). This procedure was recently applied to the quantitative estimation of the crystallizable albumin and γ -globulin in normal and pathological human cerebrospinal fluid (5). The method depends upon a calibration curve, for the antibody excess region, relating the quantity of added antigen nitrogen to the total nitrogen precipitated from a constant volume of antiserum by varying known quantities of antigen. It was, therefore, of importance to determine whether or not preparations of γ -globulin from different sources were uniform in their capacity to react with antibody. Jager, Smith, Nickerson, and Brown (10) have recently reported that two preparations of γ -globulin (II-1,2 and II-3) prepared by ethanol fractionation (11) gave identical quantitative precipitin curves with antisera, although they contained some materials of sedimentation constants higher than 7 Svedberg units. In the present study four samples of human γ -globulin, including the two samples used by Jager *et al.* (10), were found, within experimental error, to have the same reactivity with homologous antibody. These four samples represent γ_2 -globulins in the nomenclature of Deutsch *et al.* (12). A sample of γ_1 -globulin prepared in Dr. Deutsch's laboratory (12), however, showed only about 60 per cent of the capacity per unit of nitrogen to precipitate the rabbit antibody to γ_2 -globulin, although all of the antibody to γ_2 -globulin could be precipitated by addition of excess γ_1 -globulin.

EXPERIMENTAL

γ_2 -Globulins—Preparation S was the water-soluble euglobulin prepared from human serum as described by Kendall (3); this fraction was shown by him to behave as a single antigen in its reaction with homologous

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antibody. The sample showed only a single boundary electrophoretically. γ -Globulins II-1,2 and II-3 were the samples described by Jager *et al.* (10). They were prepared by ethanol fractionation (11) and were obtained from

TABLE I
Total Nitrogen Precipitated from 1.0 Ml. of Anti- γ_2 -globulin by Varying Amounts of γ_2 - and γ_1 -Globulins

The values are expressed in micrograms.

γ -Globulin N added	Anti- γ -globulin, Pool VII				γ -Globulin N added	Anti- γ -globulin, Pool IX					
	Sam- ple S	Sample B	Frac- tion II-1, 2	Frac- tion II-3		Sam- ple S	Sample B		Fraction II-1, 2		Fraction II-3
4.1	25	27		27	4.1	38					27
4.4			25		4.4		28		32		36
8.1		53, 52		52	4.5			34			
8.2	51				4.6					31	
8.7			53		8.1						52
12.2		75, 68		69	8.2	57					
12.3	70				8.7		52		56		
13.1			69		8.8						58
15.3		80			8.9			57			
16.2		84		85	9.1					54	
16.4	79				12.1						77
17.4			74*		12.3	78					
18.3		96			13.1		72		78		
20.0				98*	13.2						78
20.3		97*			13.4			77			
20.6	99				13.7				79		
21.7			98*		14.5		74				
24.4		114†			16.0			85			
					16.1						91
					16.4	97					
					17.4		91		91		
					17.6						95
					18.2					97	
					18.9						99
					19.6				105		
					20.0						107
					20.6			101			
					20.8	112					
					21.7		110*		110*		

Dr. Emil L. Smith of the University of Utah. Sample B was prepared by ethanol fractionation in the laboratories of Dr. E. J. Cohn and was supplied by Dr. E. Brand. It was found in Dr. Cohn's laboratory to contain 98.7 per cent of γ -globulin, 1.3 per cent of albumin, and a trace of

β -globulin electrophoretically. In the ultracentrifuge it showed 85 per cent globulin of sedimentation constant 7, 11 per cent of high molecular weight globulin, and 4 per cent albumin and "slow sedimenting" globulins.

TABLE I—*Concluded*

Antigen N added	Anti- γ -globulin, Pool XI							Antigen N added	Anti- γ -globulin, Pool XII	
	Sample B		Fraction II-1, 2		Fraction II-3		γ_1		Frac- tion II-3	γ_1
	2 days	7 days	2 days	7 days	2 days	7 days	2 days			
3.9					31	32		3.4		16
4.1			32	34				6.2	40	
4.3	30, 31	32, 32					21	6.9		29
7.7					48	54		10.3		40
8.1			53	53				12.3	75	
8.5	46, 53	49†, 54					35	13.8		47
10.0							42	17.2		61
11.6					68	72		18.5	91	
12.2			72	76				24.6	107	
12.8	68, 70	70, 74					49	25.8		82
15.6					85	84		34.4		102*
16.2			84	88				45.9		118†
17.0	89, 90	95, 91					58			
18.2			99	103						
19.2	102, 101	108, 104								
19.9							74			
23.0					107	114				
27.2			122††	125†						
28.8	126, 132†	134, 130†								
30.2							95			
34.5					131†	132†				
36.4			132†	128†						
38.4	136, 132†	140, 139†								
39.8							116			
46.0						138†				
50.4							126†			
60.4							127†			

Unless indicated all supernatants contained excess antibody.

* Supernatant showed neither antigen nor antibody.

† Supernatant showed slight antigen excess.

‡ One determination discarded.

|| Supernatant showed slight amount of both antigen and antibody.

γ_1 -Globulin—The sample of γ_1 -globulin was prepared in the laboratories of Dr. H. F. Deutsch and obtained through Dr. M. L. Petermann. It was found by Dr. Deutsch to show a single electrophoretic component of mobility intermediate between γ_2 - and β -globulins at pH 8.5. In the

ultracentrifuge it showed three components: 56.5 per cent of a component with a sedimentation constant of 7, corresponding to normal globulin, 21.3 per cent with a sedimentation constant of 8 to 12, and 22.2 per cent with a sedimentation constant of 20; the component having a sedimentation constant of 20 was relatively inhomogeneous.

Antisera—Antisera were prepared by injecting rabbits intravenously with alum-precipitated γ_2 -globulins B or II-3 as described previously (5). Pools of serum from several rabbits were made and absorbed by the addition of small quantities of crystalline human albumin until precipitation no longer occurred. The absorbed antiserum pools were tested by the agar diffusion method of Oudin (13), in which undiluted human serum was layered over an agar gel containing the absorbed antiserum; as diffusion of the human serum into the agar proceeded, only one band of precipitation could be observed. This corresponded to a band produced by layering γ -globulin over a similar tube of agar gel containing antiserum. These findings indicate that the absorbed antiserum pools contained antibody only to the γ_2 -globulin. Further evidence that the antisera contained antibody to only a single antigen was provided by the failure of individual supernatants from assays of human serum or cerebrospinal fluid to show a zone containing both antigen and antibody (3); in addition, the same values for γ -globulin, within experimental error, were obtained when the same spinal fluids were analyzed with several different calibrated antisera. After antisera were tested by the agar diffusion method, they were diluted so that about 100 to 150 γ of specific precipitate nitrogen per ml. would be obtained.

Quantitative Precipitin Curves—Varying quantities, in terms of nitrogen, of each of the samples of γ -globulin were added in duplicate to 1.0 ml. portions of antiserum; portions of antiserum to which no γ -globulin was added served as controls. The total volume was adjusted to 3.0 ml. with saline. The contents of the tubes were mixed, incubated for 1 hour at 37°, and placed in the refrigerator for 48 hours. The tubes were centrifuged in a refrigerated centrifuge, and the precipitates washed twice with 3.0 ml. portions of chilled saline and analyzed for nitrogen ((14), cf. (8)) by the Markham micro-Kjeldahl method (15). The serum supernatants were divided in half and tested for the presence of antigen and antibody (5).

Since it has been shown that, with small quantities of antibody nitrogen, 48 hours may not always be sufficient for maximum precipitation (14), a series of determinations was made with the tubes kept in the refrigerator for 7 days before centrifugation.

The results obtained with the various samples of four pools of rabbit anti- γ -globulin are summarized in Table I and Figs. 1 and 2. It is evident

that all four preparations of γ_2 -globulin showed the same capacity per unit of nitrogen to precipitate the rabbit antibody throughout the region of

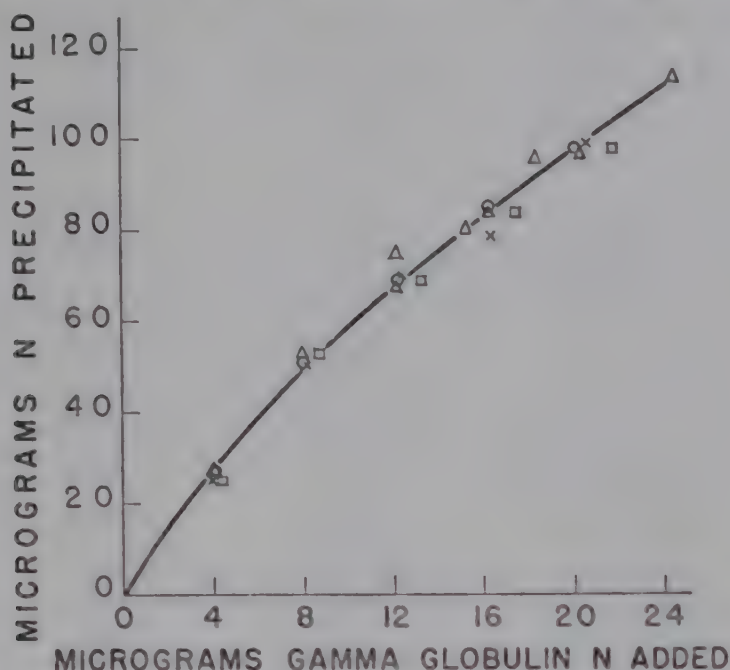


FIG. 1. Reaction of four γ_2 -globulin preparations with rabbit anti- γ -globulin Pool VII. \times = Sample S; Δ = Sample B; \square = fraction II-1,2; \circ = fraction II-3.

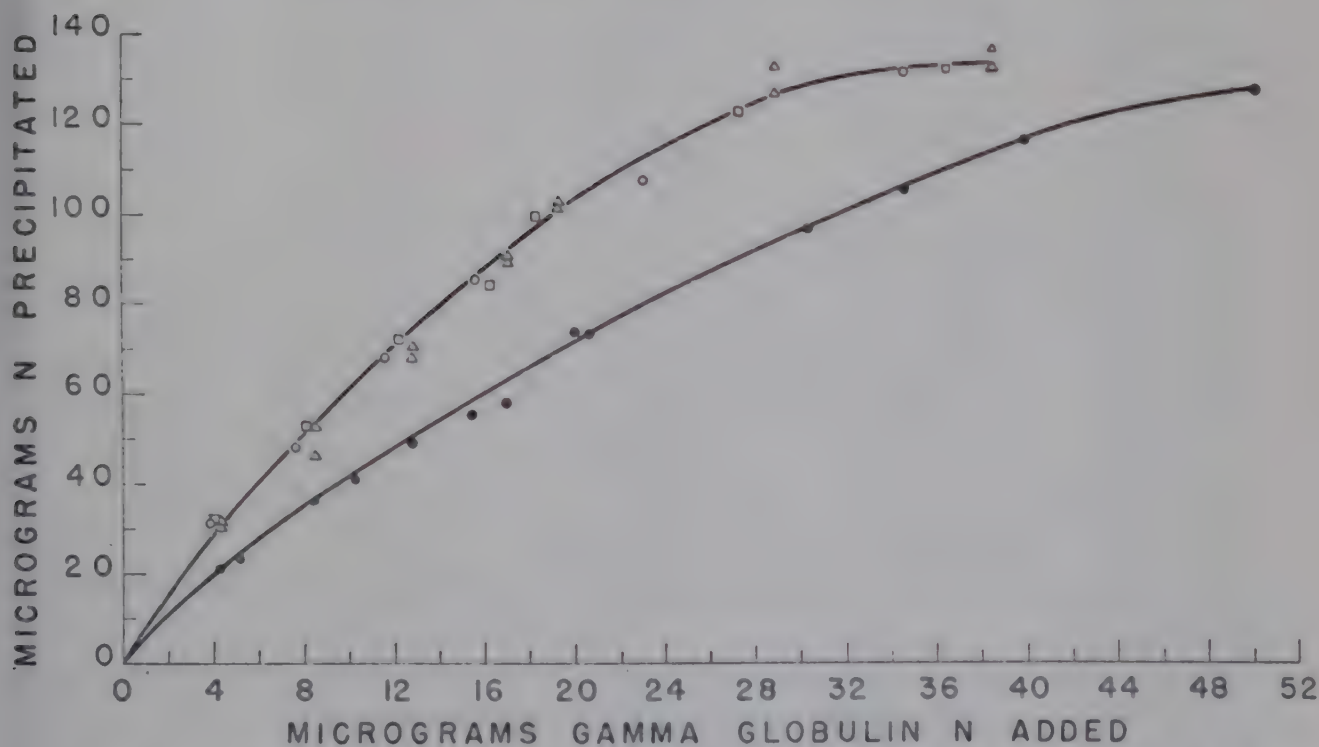


FIG. 2. Reaction of γ_2 - and γ_1 -globulins with rabbit anti- γ -globulin Pool XI. Δ = Sample B; \square = fraction II-1,2; \circ = fraction II-3; \bullet = γ_1 -globulin.

antibody excess, the equivalence zone, and in the region of slight and moderate antigen excess. Occasional variations in individual points may

be as much numerically as ± 5 to 8 per cent from the values on the curves, but variations of this magnitude occur even with repeated quantitative precipitin curves on the same γ -globulin preparations (Table I, anti- γ -globulin Pool IX). The series of determinations in which the tubes containing the antigen and serum mixtures were allowed to remain in the refrigerator for 7 days instead of 2 days (Pool XI) showed about 4 to 6

TABLE II
Precipitating Power of Mixtures of γ_1 - and γ_2 -Globulins for Rabbit Anti- γ_2 -globulin Compared with Original Fractions

γ -Globulin N added	Total N precipitated (antiglobulin Pool XI)			
	γ_1 -Globulin	31 per cent γ_2 -globulin-II-3	57 per cent γ_2 -globulin-II-3	γ_2 -Globulin-II-3
γ	γ	γ	γ	γ
3.9				31
4.3		26		
5.2	23			
5.4			29	
7.7				48
8.6		43		
10.3	41			
10.7			53	
11.6				68
12.9		56		
15.5	59			
15.6				85
16.1			68	
17.2		77		
20.6	73			
21.4			94	
23.0				107
28.6		99		
32.1			129	
34.4	105	117		
34.5				131

γ of additional total N in the precipitates for the three γ -globulin samples studied.

Table I also provides a comparison of the capacity of γ_1 -globulin to react with antiserum Pools XI and XII. It is evident from the data and from Fig. 2 that a given quantity of γ_1 -globulin N is less effective than the same quantity of γ_2 -globulin N in precipitating antibody throughout the region of antibody excess. The relative effectiveness of the two preparations in precipitating antibody may be compared by interpolation from the curves of the quantities of each required to give the same amount

of total N in the precipitates. For instance, in Fig. 2, 10.1 and 16.6 γ of γ_2 - and γ_1 -globulin gave 60 γ of total specific precipitate N and the ratio 10.1:16.6 indicates that the γ_1 -globulin was only 61 per cent as effective in precipitating with antibody as was γ_2 -globulin. Similar comparisons made at varying points throughout the antibody excess region gave an average of 61 per cent for the precipitating power of γ_1 -globulin relative to γ_2 -globulin; the data for antiserum Pool XII were computed to be about 57 per cent; this value is about the same within experimental error. Both the γ_1 - and γ_2 -globulins gave about the same quantity of specific precipitate N at the point of maximum precipitation (Table I, Fig. 2).

To explore further the reactions of the γ_1 - and γ_2 -globulins with antisera, mixtures of the γ_1 preparation and fraction II-3 were made; one mixture contained 31 per cent and the other 57 per cent γ_2 -globulin. Quantitative precipitin curves were set up with antiglobulin Pool XII. It is evident from Table II that the reactivity of the mixtures is intermediate between that of γ_1 - and γ_2 -globulins, the amount of total N precipitated by a given quantity of antigen N increasing as the proportion of γ_2 -globulin in the mixture increased.

In general, supernatant tests showed a region of antibody excess, an equivalence zone in which neither antigen nor antibody was present, and a zone of antigen excess. Occasionally, in a few tubes in the region of the equivalence zone, tests for both antigen and antibody were obtained with the γ_1 -globulin with Pool XI (Table I).

DISCUSSION

The results obtained establish the immunochemical uniformity of preparations of γ_2 -globulin prepared in three different laboratories (*cf.* (10)) and indicate that γ_2 -globulin preparations obtained by ethanol fractionation (11) (fraction II-3 or II-1,2) or by salt fractionation and dialysis, as described by Kendall (3), provide a readily reproducible primary standard for the preparation of rabbit antisera and for the immunochemical estimation of γ -globulin in biological fluids such as serum, cerebrospinal fluid, etc. (*cf.* (5, 8)). All four samples were essentially homogeneous electrophoretically, but two of them (fractions II-3 and II-1,2) contained about 75 per cent of material, with a sedimentation constant corresponding to that of normal γ -globulin, the remaining 25 per cent being composed of heterogeneous material of sedimentation constant 9 to 17 S (10). Sample B contained 85 per cent of normal γ -globulin and only 11 per cent of heavier globulin materials. Sample S was not characterized in the ultracentrifuge. Since all four preparations were of equivalent potency in precipitating rabbit antibody, it is probable that these heavier constituents also react with antibody to γ -globulin, as has been found for the corresponding com-

ponents of horse serum by Treffers, Moore, and Heidelberger (16). Were the heavy components of Sample B and fractions II-1,2 and II-3 inert immunologically, the latter two samples should have shown less capacity to precipitate antibody per unit of antigen nitrogen.

The sample of γ_1 -globulin showed a lower capacity per unit nitrogen to precipitate antibody from the antiserum pools (Table I, Fig. 2), although, within experimental error, the same maximum quantity of specific precipitate N was obtained. Computation from the curves showed the γ_1 -globulin to have 57 and 61 per cent of the precipitating power of the γ_2 samples. These values correspond closely to the value of 56.5 per cent of globulin of sedimentation constant 7 found by Deutsch *et al.* in this preparation. Absorption of the antiserum to γ_2 - with γ_1 -globulin removed all of the antibody.

There are two possible explanations for these findings. The simplest is that the γ_1 preparation of Deutsch contains about 60 per cent of γ_2 -globulin, identical immunochemically with the other γ_2 -globulin preparations, and that the remaining material is not reactive with antiserum to γ_2 -globulin and hence is immunologically unrelated. This explanation conforms very closely to the data and also satisfactorily explains the intermediate values of precipitating power found for the mixtures of γ_1 - and γ_2 -globulins. It implies, however, that the materials of higher sedimentation constant in the γ_2 preparations are immunologically similar to γ_2 -globulin, while those in the γ_1 preparation are immunologically different. At present there is no direct evidence bearing on this point except for the finding of Cohn, Deutsch, and Wetter (17) that absorption of a γ_1 antiserum with γ_2 fails to remove all of the antibody. It also suggests that the differences in mobility of the γ_1 - and γ_2 -globulins do not affect their immunological specificity.

The second possible explanation is that the reaction of γ_1 -globulin with antiserum to γ_2 -globulin is a cross-reaction. The cross-reaction would have to be of an uncommon type in that the cross-reacting antigen, if added in sufficient quantity, precipitates the same amount of antibody from the antiserum as does the homologous antigen. This type of cross-reaction appears to be relatively unusual, but data on cross-reactions are not sufficiently extensive to permit a choice between the two hypotheses. A cross-reaction in which crystalline egg albumin precipitated all of the antibody from an antiserum to the red dye, R-salt azobiphenylazo-egg albumin, has been studied (18), but in this cross-reaction the egg albumin anti-dye-egg albumin complex was very highly dissociated and differs markedly in this respect from the γ_1 -anti- γ_2 system. In view of the ultracentrifugal heterogeneity of the γ_1 -globulin, and the correspondence of the immunological reactivity with the percentage of globulin of the

usual molecular weight, the assumption of a cross-reaction appears less likely (*cf.* also (17)).

SUMMARY

1. Four samples of human γ_2 -globulin prepared in three different laboratories gave identical quantitative precipitin curves with rabbit anti- γ_2 -globulin.

2. A sample of human γ_1 -globulin showed about 60 per cent of the capacity of γ_2 -globulin to precipitate anti- γ_2 -globulin throughout the region of antibody excess, but excess of either antigen yielded the same quantity of specific precipitate nitrogen.

BIBLIOGRAPHY

1. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, **55**, 555 (1932).
2. Goettsch, E., and Kendall, F. E., *J. Biol. Chem.*, **109**, 221 (1935).
3. Kendall, F. E., *J. Clin. Invest.*, **16**, 921 (1937); *Cold Spring Harbor Symposia Quant Biol.*, **8**, 376 (1938).
4. Kabat, E. A., Heidelberger, M., and Bezer, A. E., *J. Biol. Chem.*, **168**, 629 (1947).
5. Kabat, E. A., Glusman, M., and Knaub, V., *Am. J. Med.*, **4**, 653 (1948).
6. Mazur, A., and Shorr, E., *J. Biol. Chem.*, **176**, 771 (1948).
7. Kabat, E. A., Bendich, A., and Bezer, A. E., *J. Exp. Med.*, **83**, 485 (1946).
8. Kabat, E. A., and Mayer, M. M., *Experimental immunochemistry*, Springfield (1948).
9. Gutman, A. B., *Advances in Protein Chem.*, **4**, 155 (1948).
10. Jager, B. V., Smith, E. L., Nickerson, M., and Brown, D. M., *J. Biol. Chem.*, **176**, 1177 (1948).
11. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, **68**, 459 (1946).
12. Deutsch, H. F., Alberty, R. A., and Gosting, L. J., *J. Biol. Chem.*, **165**, 21 (1946).
13. Oudin, J., *Bull. Soc. chim. biol.*, **29**, 140 (1947); *Ann. Inst. Pasteur*, **75**, 30, 109 (1948).
14. Heidelberger, M., and MacPherson, C. F. C., *Science*, **97**, 405 (1943); **98**, 63 (1943).
15. Markham, R., *Biochem. J.*, **36**, 790 (1942).
16. Treffers, H. P., Moore, D. H., and Heidelberger, M., *J. Exp. Med.*, **75**, 135 (1942).
17. Cohn, M., Deutsch, H., and Wetter, L., *J. Immunol.*, in press.
18. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, **59**, 519 (1934).

CARBOHYDRATE METABOLISM IN HIGHER PLANTS

III. BREAKDOWN OF FRUCTOSE DIPHOSPHATE BY PEA EXTRACTS*

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During the past decade evidence has been accumulating concerning the metabolism of sugars in higher plants. Because James *et al.* (1), Hanes (2), and Hassid (3) have isolated phosphorylated hexoses from plant tissues identical to those found in animal tissues, it can be assumed that the mode of transformation of sugars in plants is probably similar to that in animals. Little work, however, has been carried out to elucidate the enzyme systems involved in carbohydrate metabolism in higher plants.

Recently the enzyme aldolase (4), catalyzing the reaction that reversibly converts fructose diphosphate to triose phosphates, has been isolated from pea seeds, and its properties and kinetics have been studied. Furthermore, distribution studies (5) have revealed the presence of this enzyme in a wide variety of plants, its concentration being especially high in the meristematic tissues. Finally, the enzyme was found to be localized in the cytoplasm of leaf cells rather than in chloroplastic bodies.

The present communication will present observations on some of the enzyme systems involved in the further transformation of triose phosphates to pyruvic acid and acetaldehyde. The results indicate a striking similarity between the enzyme systems found in plants and those found in yeast and animal tissues with respect to the chemistry of the reactions and of the enzymes involved.

Preparations—Pea seeds (Dwarf Telephone) were employed as the source of the fermentation system. Since the system was stable to acetone, a large quantity of pea acetone powder was prepared as follows: Peas were soaked in distilled water at 2° for 12 hours, homogenized in 5 to 10 times their weight of acetone at 0° in a Waring blender for 10 minutes, filtered through a large Büchner funnel, and washed twice with acetone and twice with dry, peroxide-free ether. The dry, amorphous powder when stored at -10° proved to be stable for an indefinite period.

Three fermentation systems were prepared from pea acetone powder. Preparation A, acetone powder was suspended in 5 times its weight of distilled water, and adjusted to pH 6.5 with 0.1 M NaHCO₃. After 10 min-

* This paper was presented in part at the meeting of the American Chemical Society at San Francisco, March 28, 1949.

utes, the suspension was centrifuged for 10 minutes at 2500 R.P.M. and the residue discarded. The opaque supernatant solution was used directly. Preparation B was the same as Preparation A, except that the extract was dialyzed for 2 hours against 10^{-5} M thioglycolate at 4°. A magnetic stirrer was employed to agitate the dialyzing fluid vigorously. Preparation C was the same as Preparation B, except that dialysis was carried out for 12 hours under the same conditions. No attempts were made to separate or isolate the various enzyme systems.

Methods—Conventional Warburg manometric techniques were employed in measuring oxygen uptake and carbon dioxide formation. Fructose diphosphate (FDP) was estimated by first converting it completely to triose phosphates by a pea aldolase-sulfite mixture and then measuring the triose phosphates by their sensitivity to mild alkaline hydrolysis (4). Phosphoglyceric acid was estimated from the difference between total phosphate and phosphate released after 3 hours hydrolysis in 1 N HCl at 100°, phosphopyruvic acid by phosphate released after iodine-NaOH oxidation (6), and pyruvic acid by the Friedemann-Haugen procedure (7). In estimating mixtures of phosphopyruvic acid and adenosine di- and triphosphates, clear cut separations of phosphopyruvic acid from the adenosine derivatives were effected by barium fractionation as described by Umbreit *et al.* (6). Inorganic phosphate was measured by the Fiske and Subbarow method (8) and organic phosphate by the perchloric acid digestion procedure (9).

Reagents—Acid barium FDP of 85 per cent purity by aldolase analysis was prepared by the method of Neuberg *et al.* (10), from barium FDP obtained from the Schwarz Laboratories, Inc. In aqueous solutions, however, the concentration of FDP decreased after a week, presumably by hydrolysis to fructose-6-phosphate, notwithstanding storage at 0°. Therefore, for quantitative experiments, only freshly prepared solutions of sodium FDP were used. Adenylic acid and adenosine diphosphate were obtained from the Sigma Chemical Company, and adenosine triphosphate (Na_4) from the Rohm and Haas Company. Coenzyme I was prepared from bakers' yeast by the method of LePage (11) and assayed 44 per cent spectrophotometrically. We are indebted to Dr. H. A. Barker for generous samples of acid barium 3-phosphoglycerate and silver barium phosphopyruvate.

Results

$\text{FDP} \rightleftharpoons \text{Phosphoglyceric Acid}$

As summarized in Fig. 1, when FDP is added to Preparation C, no acid (measured as CO_2 in a bicarbonate medium) is formed anaerobically unless coenzyme I, arsenate, and a suitable oxidant are added. Both coenzyme

I and arsenate are specifically required. Inorganic phosphate with or without adenylic acid is inert in the system in so far as the rapid formation of acid is concerned. A variety of oxidants may be employed to complete the system. It is important to point out that there is a slow formation of acid in the presence of inorganic phosphate. This effect is related to a phosphatase which slowly hydrolyzes 1,3-diphosphoglyceric acid formed during the fermentation. Finally, fluoride was employed to limit the breakdown of FDP to phosphoglyceric acid.

With these factors in mind, the study was resolved into a series of experiments designed to demonstrate the component enzymes involved in

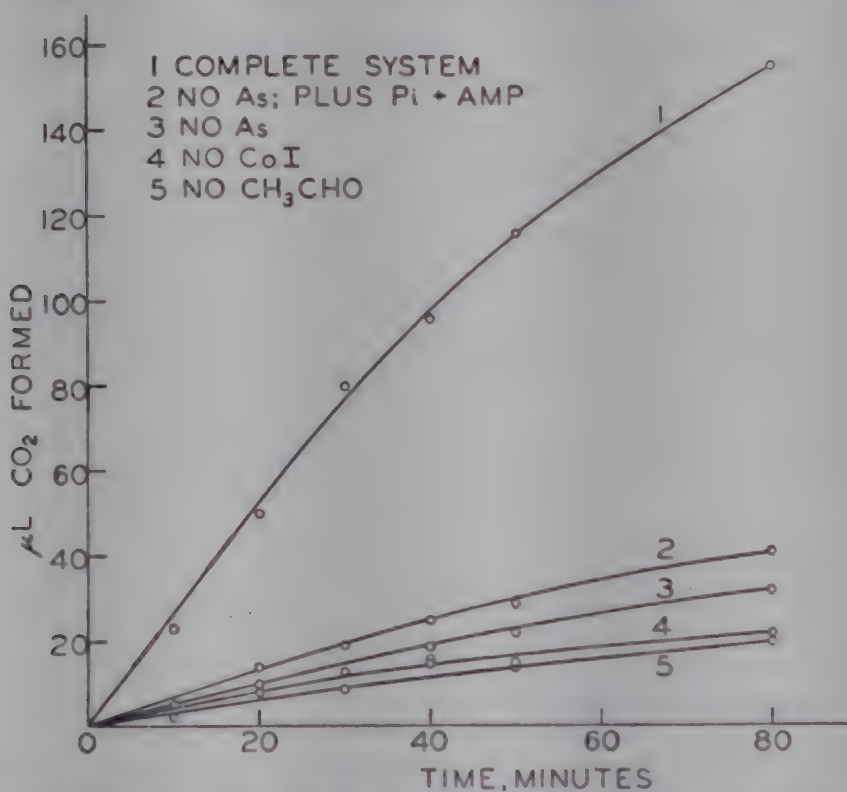


FIG. 1. Breakdown of FDP as affected by components of the system. The conditions of the experiment are described in the legend of Table I.

the fermentation system. The components proved to be (1) aldolase, (2) isomerase, (3) triose phosphate dehydrogenase, (4) a phosphotransferase (?), and (5) the adjuncts coenzyme I, arsenate, and a suitable oxidant.

The first enzyme system, aldolase, has already been defined in Paper I of this series (4). It cleaves FDP to dihydroxyacetone phosphate and 3-phosphoglyceraldehyde, the equilibrium constant at 37° being about 5×10^{-3} mole per liter. Therefore, on addition of FDP to Preparation C an equilibrium mixture between FDP and the two triose phosphates is rapidly established. As in animal tissues and yeast, an isomerase is also present in Preparation C, which catalyzes an equilibrium between the two triose phosphates (see the scheme in the "Discussion"). The proof for

this conclusion, while not as direct as the actual isolation of isomerase would be, is clearly demonstrated in the following considerations. First, in a complete fermentation system, it is obvious that only if an isomerase is present can 1 equivalent of FDP yield 2 equivalents of acid. Table I summarizes the data of CO₂ measurements in which the ratio of 1 equivalent of FDP utilized to 2 equivalents of acid formed is demonstrated. This ratio could not have been realized if isomerase had been absent, since of the two triose phosphates formed from the aldolase cleavage of FDP only 3-phosphoglyceraldehyde would have been oxidized by triose phosphate dehydrogenase to yield phosphoglyceric acid. The other triose phosphate, dihydroxyacetone phosphate, would have accumulated, since it would not be in equilibrium with phosphoglyceraldehyde and is itself not

TABLE I

Formation of Phosphoglyceric Acid from Fructose Diphosphate

Each manometric cup contained 1 cc. of enzyme (Preparation C), different concentrations of fructose diphosphate, 0.05 cc. of 0.05 M arsenate, 0.1 cc. of 0.1 per cent coenzyme I, 0.5 cc. of 0.05 M sodium bicarbonate, 0.1 cc. of M acetaldehyde, 0.5 cc. of 0.1 M fluoride, and water to a total volume of 3 cc. Temperature 37°; gas phase 95 per cent N₂-5 per cent CO₂.

Fructose diphosphate added	CO ₂ formed	Triose phosphate formed	Phosphoglyceric acid formed
μM	μM	μM	μM
4.3	8.1	0	7.6
4.4	8.6	0	8.1
4.4*	0	2.1	0.1

* 0.1 μM of iodoacetamide added.

attacked by triose phosphate dehydrogenase. It also follows that on addition of iodoacetamide, which strongly inhibits triose phosphate dehydrogenase, there should be an accumulation of an equilibrium mixture of 97 per cent dihydroxyacetone phosphate and 3 per cent phosphoglyceraldehyde, provided isomerase is present. If, however, isomerase is absent in Preparation C, then an equimolar mixture of the two triose phosphates should be demonstrated. Analyses of the iodoacetamide-treated fermentation system with FDP as the initial substrate showed the presence of 96 per cent dihydroxyacetone phosphate and 4 per cent phosphoglyceraldehyde instead of a 50:50 mixture, which occurs only if isomerase is absent as is the case in purified aldolase preparations (4). Finally, in the absence of a suitable oxidant such as acetaldehyde or methylene blue, 2 equivalents of coenzyme I must be required to oxidize 2 equivalents of triose phosphates derived from FDP through the agency of aldolase and isomer-

ase. Fig. 2 indicates that this condition is completely fulfilled. The evidence therefore would appear to justify the conclusion that an isomerase occurs in Preparation C which presents triose phosphate dehydrogenase with 2 equivalents of triose phosphate from 1 equivalent of FDP.

The third enzyme, triose phosphate dehydrogenase, is strikingly similar to its counterpart found in yeast and animal tissues. The components of the system include coenzyme I and arsenate. An oxidant is also required to regenerate reduced coenzyme I. It is reversibly inhibited by copper and irreversibly by iodoacetamide.

The identification of triose phosphate dehydrogenase resolved itself into three considerations, (1) the oxidation-reduction of coenzyme I, (2) the

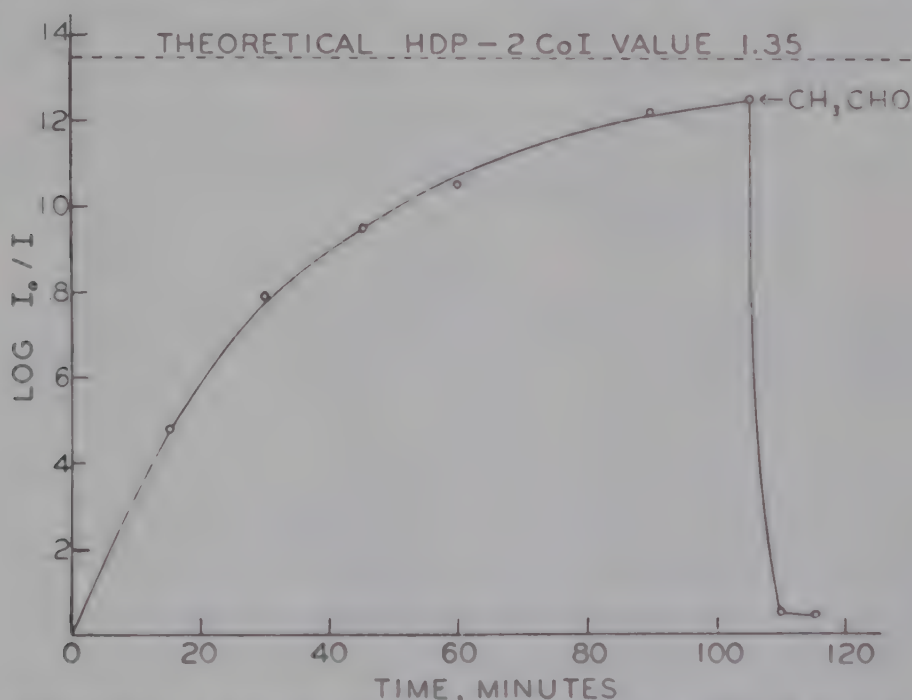


FIG. 2. Reduction of 2 equivalents of coenzyme I by 1 equivalent of FDP. Absorption at $340 \text{ m}\mu$ measured with the Beckman spectrophotometer.

rôle of arsenate and phosphate in the oxidation, and (3) the effect of inhibitors.

Oxidation-Reduction of Coenzyme I—As is indicated in Fig. 3, in the presence of arsenate, triose phosphate (derived from FDP), and triose phosphate dehydrogenase (Preparation C), coenzyme I is reduced to dihydrocoenzyme I, with its characteristic absorption band at $340 \text{ m}\mu$. Further, as indicated in Fig. 2, in the presence of alcohol dehydrogenase, which is found in rather large concentrations in Preparation C, the reoxidation of dihydrocoenzyme I can be readily coupled with the reduction of acetaldehyde to ethyl alcohol. Table II summarizes the data characterizing pea alcohol dehydrogenase.

Pyruvic acid can serve indirectly as a suitable oxidant. Since lactic de-

hydrogenase is absent in Preparation C, reduction of pyruvic acid to lactic acid cannot be coupled with the reoxidation of dihydrocoenzyme I. However, because of the presence of an active carboxylase in Preparation C, pyruvic acid serves as a source of acetaldehyde.

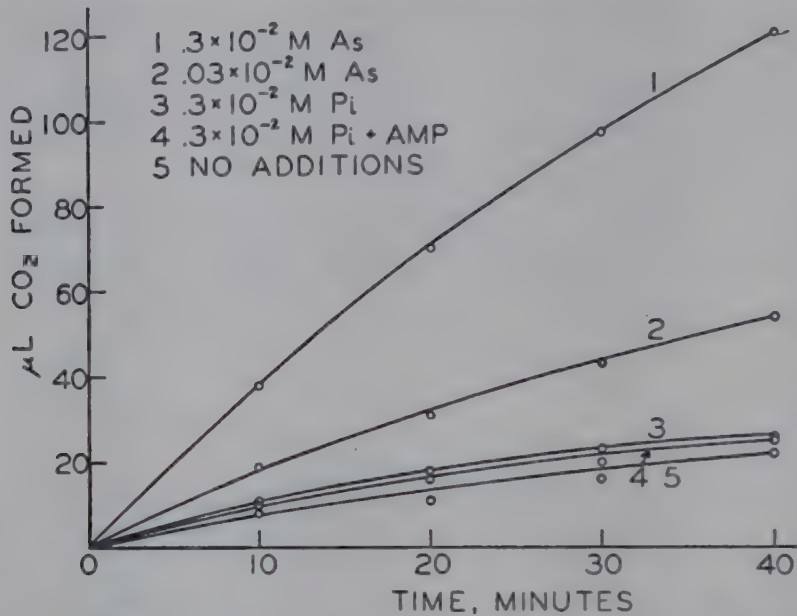


FIG. 3. Effect of different concentrations of arsenate (*As*) and phosphate (*Pi*) on the fermentation of FDP.

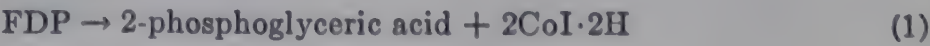
TABLE II

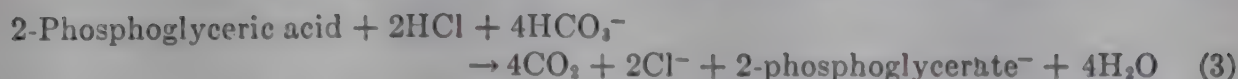
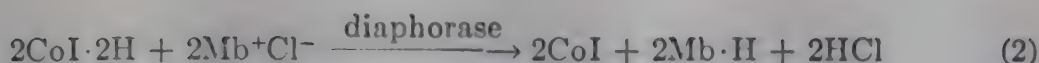
Factors Affecting Activity of Pea Alcohol Dehydrogenase

Each manometric cup contained 1 cc. of enzyme (Preparation C), 0.2 cc. of 0.1 M ferricyanide, 0.5 cc. of 0.05 M sodium bicarbonate. As indicated, additions were 0.1 cc. of 0.1 per cent coenzyme I, 0.1 cc. of different absolute alcohols, and 0.1 cc. of 0.001 M iodoacetamide. Water was added to a total volume of 3 cc. Temperature 37°; gas phase 95 per cent N₂-5 per cent CO₂.

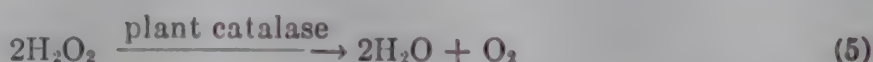
Additions	CO ₂
	μl. per 20 min.
Enzyme + ethyl alcohol + coenzyme I.....	65
“ + “ “ no “ “.....	1
“ + propyl alcohol + “ “.....	30
“ + butyl “ + “ “.....	0
“ + ethyl “ + “ “ + iodoacetamide.....	0

Methylene blue can replace acetaldehyde provided pea diaphorase is present. Preparation B contains an active diaphorase which catalyzes the oxidation of reduced coenzyme I. Thus in the presence of a bicarbonate buffer, the following reactions account for the formation of CO₂ as measured in a Warburg manometer.





Furthermore, since methylene blue is autoxidizable, oxygen can be coupled to the system



Therefore, in theory, for each mole of FDP utilized 1 mole of oxygen should be consumed, as indicated by Equations 1, 2, 4, and 5, which together yield the over-all equation



The data supporting this series of reactions are presented in Table III.

TABLE III

Balance Sheet Studies in Breakdown of Fructose Diphosphate \rightarrow 2-Phosphoglyceric Acid

Each manometric cup contained 1 cc. of enzyme, a given concentration of fructose diphosphate, 0.1 cc. of M acetaldehyde, 0.5 cc. of 0.05 M sodium bicarbonate, 0.05 cc. of 0.05 M arsenate, and 0.5 cc. of 0.1 M fluoride. Where indicated 0.1 cc. of 0.05 M methylene blue was employed. Water was added to a final volume of 3 cc.; gas phase 95 per cent N_2 -5 per cent CO_2 ; temperature 37° . In oxygen uptake experiments, 0.1 cc. of 0.1 per cent methylene blue was employed, and the center well contained 0.2 cc. of 10 per cent sodium hydroxide. Gas phase air; temperature 37° .

Fructose diphosphate μM	Enzyme preparation	CO_2				O_2	
		CH_3CHO		Methylene blue		Theory	Experimental
		Theory	Experimental	Theory	Experimental		
		c. mm.	c. mm.	c. mm.	c. mm.	c. mm.	c. mm.
2	A	90	87	180	180	45	41
2.5	B	112	101				
3	"	134	115				
4	C	182	186				
5	Green peas					112	103
2	Yeast extract*	90	80	180	150		

* Prepared according to the method of Neuberg and Lustig (15).

Ferricyanide can also be employed, though it appears to be somewhat toxic to the system. Being a strong oxidant, it probably oxidizes the SH groups of triose phosphate dehydrogenase to the disulfide or inactive form. α -Ketoglutaric acid and diacetyl do not replace acetaldehyde.

Rôle of Arsenate and Phosphate—Manometric studies revealed that the addition of phosphate to either Preparation A, B, or C with or without adenylic acid does not lead to the formation of acid. However, an arsenate effect was consistently observed with the addition of suitable concentrations of the anion (Fig. 3). Spectrophotometric data revealed that coenzyme I was reduced in the presence of either phosphate or arsenate; in the absence of these anions there was no reduction (Fig. 4). The results indicated that either arsenate or phosphate is involved in the oxidation of 3-phosphoglyceraldehyde. However, whereas 1-arseno-3-phosphoglyceraldehyde breaks down spontaneously (12), the corresponding 1,3-phospho complex cannot be coupled with available phosphate acceptors, adenosine monophosphate (AMP) and adenosine diphosphate (ADP), but is slowly

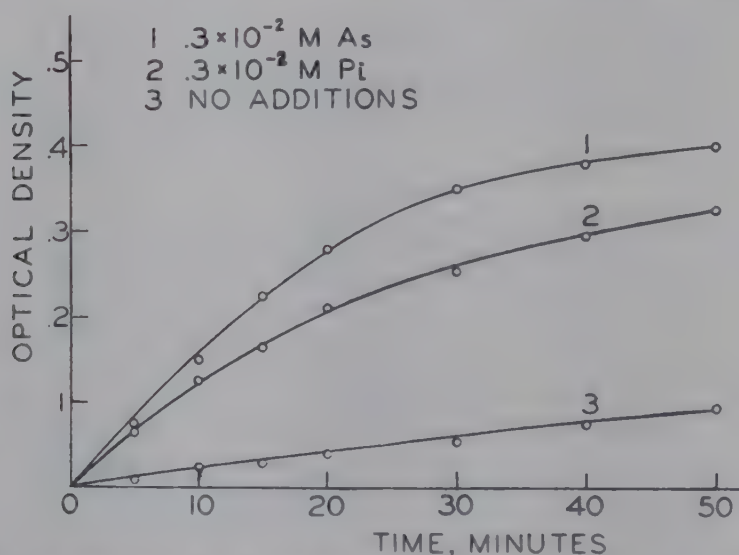


FIG. 4. Effect of arsenate (As) and phosphate (Pi) on the reduction of coenzyme I by FDP + fermentation system.

hydrolyzed. With phosphate, therefore, either a highly specific phosphate acceptor is missing or a specific transphosphorylase has been inactivated. These results can be duplicated with any of the three preparations and with a preparation made from fresh green peas (adenosine triphosphatase is present in all preparations). Table IV summarizes the data.

Inhibitors—The effects of several reagents were investigated and are listed in Table V. Cyanide was excluded from the list since it combined with triose phosphates to form cyanohydrins. Pea triose phosphate dehydrogenase resembles closely its counterpart in yeast and animal tissues in that traces of copper inactivate the system reversibly. This inhibition is reversed by the addition of cysteine, thioglycolate, or glutathione. Iodoacetamide inactivates the system irreversibly, since sulfhydryl reagents do not reverse the inhibition. It is therefore obvious from a comparative point of view that the integrity of the SH groups of the protein portion of

triose phosphate dehydrogenase derived from animal (13), yeast (12), or plant sources must be maintained for activity.

TABLE IV

Effect of Arsenate and Phosphate on Breakdown of Fructose Diphosphate

Same conditions as in the legend of Table I. Concentrations of arsenate and phosphate, 10 μM .

Additions	CO ₂ per 15 min.	
	Preparation A	Preparation C
	$\mu\text{l.}$	$\mu\text{l.}$
Arsenate + FDP*	63	55
Phosphate	6	10
“ + FDP	7	13
“ + AMP†	4	
“ + “ + FDP	13	13
“ + ADP‡	5	
“ + “ + FDP	11	14
“ + ATP§	7	
“ + “ + FDP	14	12

* Fructose diphosphate (5 μM).

† Adenylic acid (10 μM).

‡ Adenosine diphosphate (10 μM).

§ Adenosine triphosphate (10 μM).

TABLE V

Effect of Inhibitors on Breakdown of Fructose Diphosphate

Conditions as in the legend of Table I.

Inhibitor	Final concentration	Degree of inhibition
	<i>mole</i>	<i>per cent</i>
Azide	10^{-2}	0
Iodoacetamide	10^{-2}	100
“	10^{-3}	81
Cu	10^{-3}	100
“	10^{-4}	57
Fluoride	10^{-2}	0
Dinitrophenol	10^{-3}	7
2,4-Dichlorophenoxyacetic acid	10^{-3}	0
Nicotinamide	10^{-3}	0

Phosphoglyceric Acid \rightarrow Pyruvic Acid

When phosphoglyceric acid is added to Preparation B, little if any CO₂ formation is observed manometrically. However, on addition of magnesium (or manganese), cocarboxylase, and adenylic acid, activity is re-

stored. The addition of fluoride causes virtually complete inhibition of CO_2 formation. These results are summarized in Fig. 5. The data can be interpreted to mean that (1) an enolase is present which is sensitive to fluoride and requires magnesium for activation, (2) a phosphotransferase is present in Preparation B which transfers phosphate from phosphopyruvic acid to adenylic acid, and (3) a pyruvic acid carboxylase is activated by cocarboxylase to decarboxylate pyruvic acid to acetaldehyde and CO_2 which is measured manometrically. No additional observations were carried out on the properties of pyruvic carboxylase since the enzyme has been clearly defined (14).

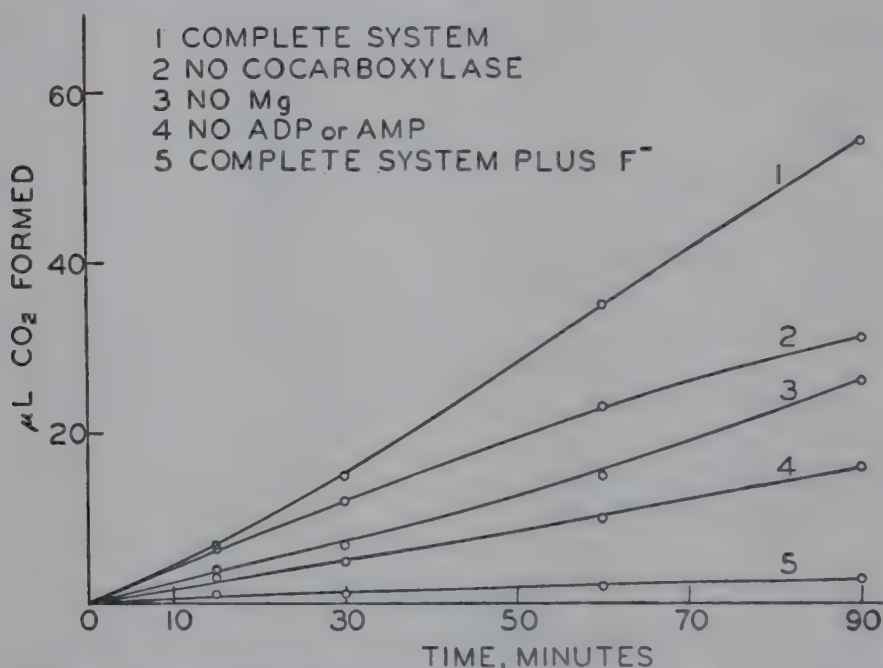


FIG. 5. Breakdown of phosphoglyceric acid as affected by components of the system. The conditions of the experiment are described in the legend of Table VI.

No further work was carried out with enolase. As is indicated in Table VI, it was found that, while Preparations A and B possess phosphotransferase, Preparation C is devoid of the enzyme. It appeared that during prolonged dialysis (the procedure used to obtain Preparation C) the phosphotransferase became inactivated. Activity could not be restored by adding divalent cations or yeast juice. Therefore, by employing Preparation C it was possible to demonstrate (1) the accumulation of phosphopyruvic acid, (2) the inability of the preparation to synthesize adenosine diphosphate from adenylic acid, and (3) the absence of pyruvic acid. However, in Preparation B, pyruvic acid accumulated in the absence of added cocarboxylase, and adenosine diphosphate was formed from adenylic acid. These conclusions were confirmed when phosphopyruvic acid was added to Preparation B in the presence of adenylic acid. Adenosine diphosphate was synthesized and CO_2 was formed (decarboxylation of pyruvic acid);

TABLE VI

Balance Sheet for Conversion of 3-Phosphoglyceric Acid to Pyruvic Acid

Each test-tube contained 1 cc. of enzyme, a given concentration of phosphoglyceric acid, 0.1 cc. of 0.1 per cent magnesium sulfate, and water to a total volume of 3 cc. In the cases indicated, the additions were 10 μM of AMP or 0.3 cc. of 0.1 M fluoride. In experiments of CO_2 formation, the manometric cups contained reagents as above and in addition 0.1 cc. of 0.1 per cent cocarboxylase and 0.5 cc. of 0.1 M citrate at pH 6.0. Temperature 37°; gas phase air.

Additions	Phosphoglyceric acid added μM	ΔP_i formed μM	Phosphopyruvic acid formed μM	Pyruvic acid formed μM	CO_2 formed μM
Preparation B + AMP.....	10	5.1	0.2	7.7	
“ “	10	0	1.3	(2.9)*	
“ “ + fluoride.....	10	0	0.1	0.3	
“ C + AMP.....	5	0	1.4	(1.6)*	
“ “ + fluoride.....	5	0	0.2	0.1	
			Added phosphopyruvic acid μM		
“ B + AMP.....			2		1.8
“ “ no AMP.....			2		0
“ C + AMP.....			2		0.1
“ “ no AMP.....			2		0

* The 2,4-dinitrophenylhydrazine method of Friedemann and Haugen does not distinguish between phosphopyruvic and pyruvic acids.

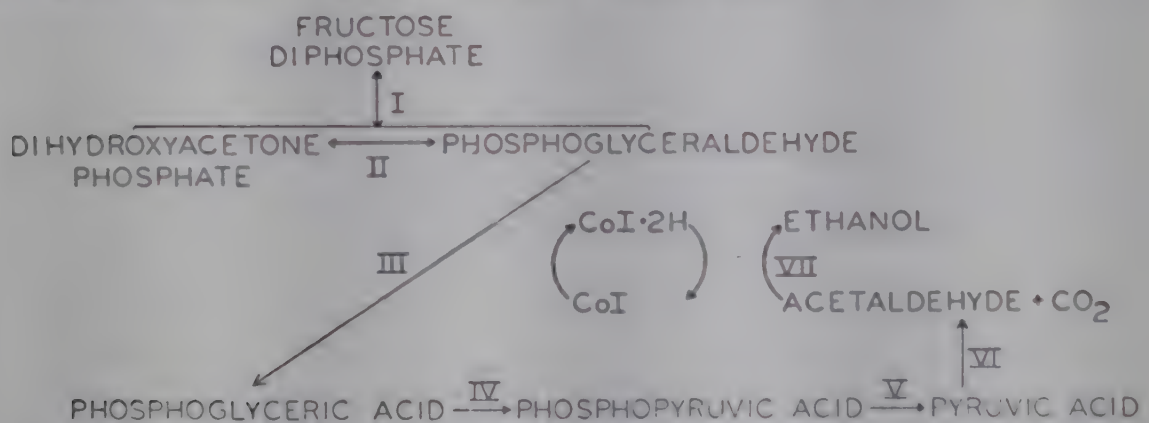


FIG. 6. Scheme for the breakdown of fructose diphosphate by pea seed extract. (I) aldolase; (II) isomerase; (III) triose phosphate dehydrogenase plus arsenate; (IV) phosphoglyceromutase plus enolase and Mg^{++} ; (V) phosphopyruvic transphosphorylase plus AMP; (VI) pyruvic carboxylase plus Mg^{++} and cocarboxylase; (VII) alcohol dehydrogenase.

in the absence of adenylic acid, no CO_2 formation could be detected. If Preparation C was employed, no changes could be observed either in the presence or absence of adenylic acid.

DISCUSSION

Fig. 6 summarizes the observations presented in this communication.

A careful analysis of each enzyme system involved in the pea fermentation system may possibly bring to light dissimilarities, but the over-all series of reactions from FDP to acetaldehyde appears to be analogous in plant and yeast cells.

SUMMARY

In the enzymic breakdown of FDP in plant tissue, the enzymes aldolase, isomerase, triose phosphate dehydrogenase, enolase, phosphotransferase, and carboxylase have been demonstrated to be involved. The results indicate that in pea seeds the fermentation of FDP is apparently similar to that in yeast and animal tissues.

BIBLIOGRAPHY

1. James, W. O., Heard, C. R. C., and James, G. M., *New Phytologist*, **43**, 62 (1944).
2. Hanes, C. S., *Proc. Roy. Soc. London, Series B*, **129**, 174 (1940).
3. Hassid, W. Z., *Plant Physiol.*, **13**, 641 (1938).
4. Stumpf, P. K., *J. Biol. Chem.*, **176**, 233 (1948).
5. Tewfik, S., and Stumpf, P. K., *Am. J. Bot.*, in press.
6. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric techniques and related methods for the study of tissue metabolism*, Minneapolis, 169 (1945).
7. Freidemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147**, 415 (1943).
8. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
9. King, E. J., *Biochem. J.*, **26**, 292 (1932).
10. Neuberg, C., Lustig, H., and Rothenberg, M. A., *Arch. Biochem.*, **3**, 33 (1943-44).
11. LePage, G. A., *J. Biol. Chem.*, **168**, 623 (1947).
12. Warburg, O., and Christian, W., *Biochem. Z.*, **303**, 40 (1939).
13. Cori, G. T., Slein, M. W., and Cori, C. F., *J. Biol. Chem.*, **173**, 605 (1948).
14. Vennesland, B., and Felsher, R. F., *Arch. Biochem.*, **11**, 279 (1946).
15. Neuberg, C., and Lustig, H., *Arch. Biochem.*, **1**, 191 (1942-43).

PURIFICATION OF THE ENZYME RESPONSIBLE FOR THE CLEAVAGE OF CYSTATHIONINE*

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The enzyme responsible for the cleavage of cystathionine by liver tissue of rats (2-4) has been obtained in a state of considerable purification. In the course of the purification, the enzymes responsible for the formation of ammonia from serine (5) and homoserine (6) were lost but the activity in the production of hydrogen sulfide from cysteine (7) was retained. It is to be emphasized that little or no ammonia was formed by the action of the purified enzyme on cysteine or cystathionine; the possibility that DL-serine or DL-homoserine were attacked without the release of ammonia has not been investigated.

EXPERIMENTAL

Methods of Assay—1 ml. of the preparation of enzyme was incubated with 0.02 mM of substrate (0.04 mM in the case of optically inactive compounds) in a total volume of 10 ml. of 0.02 M phosphate buffer, pH 7.4. The solutions contained 0.005 M MgCl_2 and, unless otherwise indicated, 0.001 M NaCN. Cysteine was determined in a trichloroacetic filtrate by the method of Sullivan and Hess (8); ammonia was determined by the method of Archibald (9). Hydrogen sulfide formed by the action of the enzyme on cysteine was determined by aeration (with nitrogen) into cadmium acetate, followed by iodometric titration of the cadmium sulfide (10). It is to be emphasized that, due to reactions of the sulfide with cystine with the loss of sulfide as free sulfur (10), the values for hydrogen sulfide are to be considered as minimal. In all the results reported here, the time of incubation was 30 minutes and the incubations were at 37°.

Purification of Enzyme—In a typical preparation, 40 gm. of fresh liver tissue (from six adult rats) were homogenized for 2 minutes in a Waring blender with 200 ml. of cold physiological saline. The homogenate was transferred to a 250 ml. centrifuge bottle and placed in a water bath maintained at 55-58°; as soon as the temperature of the homogenate reached 55°, the time was noted and the treatment with heat was continued for

* These studies were supported by a grant from the United States Public Health Service. A preliminary report has been presented (1).

5 minutes. The bottle was transferred to an ice-salt bath and was cooled, with stirring, until the temperature fell below 5°. The mixture was centrifuged in the cold room at 3000 R.P.M. for 30 minutes and the clear supernatant (about 120 ml.) was decanted into a 250 ml. centrifuge bottle. To 100 ml. of the supernatant were added 30 ml. of cold (4°) absolute ethanol, slowly and with stirring, and the mixture was centrifuged. The supernatant solution was decanted into a 250 ml. centrifuge bottle and an additional 50 ml. of ethanol were added. The precipitate was retained and the supernatant solution was discarded. The precipitate was dissolved in 100 ml. of physiological saline and 30 ml. of cold chloroform were added. The mixture was shaken for 5 minutes in the cold room and was then centrifuged at high speed for 30 minutes. The clear supernatant was removed by suction and a second fractionation with ethanol was performed. The precipitate obtained by the addition of 0.8 volume of ethanol was dissolved in 50 ml. of saline and the treatment with heat was repeated. The insoluble material was removed by centrifugation or filtration. This solution contained approximately 60 per cent of the activity present in the supernatant of the heat-treated homogenate and represented, on the basis of activity per mg. of nitrogen, a purification of approximately 40-fold. Approximately 20 to 30 mg. of protein, based on the nitrogen content, were present in the final solution. In the majority of cases, the addition of ethanol, slowly and with stirring, to the point of faint turbidity, followed by 6 to 8 hours in the cold room, brought about the precipitation of a product which appeared globular when viewed in ordinary light, but was seen to be octahedral crystals when viewed in polarized light. Physical studies are under way and will be reported in the near future.

It has been found, in agreement with Fromageot *et al.* (7), that the liver of the dog is an excellent source of the enzyme. Pig and cattle livers were found to be low in activity, and considerable difficulty has been experienced in attempts to use these livers as sources of the enzyme.

While the procedure outlined above has given completely satisfactory results, the following precautions, applicable in attempts at further purification, are to be noted. The enzyme was found to be unstable in aqueous solutions and was destroyed by freezing. If, during the precipitations with ethanol, the temperature was allowed to fall below 0°, considerable inactivation resulted. Tissues, frozen in the dry ice box, were without activity. Acetone powders, prepared from the homogenate, retained activity for many weeks. The solutions of the purified material in saline lost approximately half of their activity in 3 days when stored at 4°.

Activity of Purified Enzyme—The activity of the enzyme at various stages of purification is summarized in Table I. Activities are expressed as mg. of cysteine released per mg. of protein nitrogen during the 30 minutes of incubation. Cyanide was included in each digest.

The relative activities of various preparations toward DL-serine, DL-homoserine, L-cysteine, and L-cystathionine are summarized in Table II. As stated above, the most highly purified preparation did not produce ammonia from any of the substrates. It is to be seen that the relative activities toward cysteine and cystathionine were maintained throughout the

TABLE I
Purification of Enzyme

The procedure of purification and methods of assay are described in the text.

Step	Activity per mg. N	Total activity
	<i>units</i>	<i>units</i>
1. Homogenized with saline.....	0.05	101
2. Heat treatment.....	0.32	62
3. Ethanol fractionation.....	1.1	55
4. Chloroform treatment.....	7.0	48
5. Ethanol fractionation and heat treatment.....	13	40
6. Crystallization.....	17	34

TABLE II
Specificity of Purified Enzyme

The activities are expressed as mM of product obtained by the incubation of two preparations of the enzyme with the various substrates. The methods of assay are described in the text.

Substrate	Heat-treated homogenate, Step 2, Table I		Ethanol fractionation, Step 5, Table I
	Ammonia	Cysteine or hydrogen sulfide	Cysteine or hydrogen sulfide
	<i>mM</i>	<i>mM</i>	<i>mM</i>
L-Cystathionine.....	0.0088	0.0074	0.0013
+ 0.001 M NaCN.....	0.0026	0.0084	0.0072
L-Cysteine.....	0.0010	0.0056	0.0052
+ 0.001 M NaCN.....	0.0006	0.0054	0.0044
DL-Serine.....	0.0006		
+ 0.001 M NaCN.....	0.0004		
DL-Homoserine.....	0.0054		
+ 0.001 M NaCN.....	0.0004		

purification and that the activity toward cysteine was of the same order as the activity toward cystathionine. The effects of cyanide deserve some explanation. It has been reported previously (2) that cyanide, of somewhat higher concentration than used here, inhibited cysteine desulfhydrase and, consequently, increased the yield of cysteine from cystathionine. Our results may be interpreted as evidence that the purified enzyme is acti-

vated by dilute cyanide (0.001 M) and by cysteine; thus, only in the case of cystathionine would the activation by cyanide be demonstrable. In a study of inhibitors of the enzyme it has been found that cyanide of a higher concentration (0.01 M) is much less effective in the activation of the purified enzyme toward cystathionine and that there is an inhibition of the activity toward cysteine. Thus, when preparations treated with 0.01 M cyanide are compared with untreated preparations, there is an acti-

TABLE III
Effects of Cyanide

The effects of cyanide were tested with the purified preparation. Methods of assay were as described in the text. 4 γ of protein nitrogen were present in each ml. of digest.

Concentration of cyanide	Activity	
	Cystathionine	Cysteine
	<i>mM cysteine</i>	<i>mM H₂S</i>
None.....	0.0016	0.0052
0.00001 M.....	0.0031	0.0052
0.001 M.....	0.0079	0.0051
0.001 “.....	0.0072	0.0049
0.01 M.....	0.0027	0.0019

TABLE IV
Absence of Phosphate Effect

The activity of the purified preparation was tested as described in the text with L-cystathionine as the substrate. The method of Sullivan was used to measure the activity.

Digest	Activity
	<i>mg. cysteine</i>
0.02 M phosphate, pH 7.8.....	1.15
0.02 “ veronal, pH 7.8.....	1.26
Veronal + 30 mg. sodium adenosine triphosphate.....	1.06

vation toward cystathionine and an inhibition toward cysteine as originally reported. Such a study is summarized in Table III. Since the effect of activation by dilute cyanide was increased as purification proceeded, it is quite possible that the effects of the lower concentrations of cyanide are in the removal of traces of heavy metals picked up as contaminants during the isolation; cysteine would serve a similar purpose.

It is apparent from the studies summarized in this report that the purified preparation, active in the cleavage of cystathionine and in the

production of hydrogen sulfide from cysteine, does not bring about the formation of ammonia and keto acids from these substrates. Thus, the conflict in the results, discussed by Fromageot (11) as to whether the product of the desulfuration of cysteine is ammonia plus pyruvic acid or is another product, possibly alanine, is probably due to differences in the purity of preparation of the enzyme.

Absence of Phosphate Effect—It has been reported (3) that adenosine triphosphate was required for the activity of the enzyme. In Table IV, the activity of the enzyme toward cystathionine in phosphate buffer, in barbiturate buffer, and in the presence of adenosine triphosphate is summarized. It is obvious that neither inorganic phosphate nor adenosine triphosphate is required for the activity of the enzyme.

SUMMARY

The enzyme responsible for the cleavage of the cystathionine by liver tissues of rats has been isolated in a state of considerable purification. The purified enzyme does not produce ammonia from DL-serine or DL-homoserine, but does produce hydrogen sulfide from cysteine. It appears probable that the enzyme is identical with cysteine desulfurase (desulfhydrase). Neither adenosine triphosphate nor inorganic phosphate is required for the activity of the enzyme.

BIBLIOGRAPHY

1. Binkley, F., *Federation Proc.*, **8**, 184 (1949).
2. Binkley, F., Anslow, W. P., Jr., and du Vigneaud, V., *J. Biol. Chem.*, **143**, 559 (1942).
3. Binkley, F., *J. Biol. Chem.*, **155**, 39 (1944).
4. Anslow, W. P., Jr., and du Vigneaud, V., *J. Biol. Chem.*, **170**, 245 (1947).
5. Binkley, F., *J. Biol. Chem.*, **150**, 261 (1943).
6. Carroll, W. R., Stacy, G. W., and du Vigneaud, V., *J. Biol. Chem.*, **180**, 375 (1949).
7. Fromageot, C., Wookey, E., and Chaix, P., *Compt. rend. Acad.*, **209**, 1019 (1939).
8. Sullivan, M. X., and Hess, W. C., *J. Biol. Chem.*, **116**, 221 (1936).
9. Archibald, R. A., *J. Biol. Chem.*, **154**, 657 (1944).
10. Smythe, C. V., *J. Biol. Chem.*, **142**, 387 (1942).
11. Fromageot, C., *Advances in Enzymol.*, **7**, 369 (1947).

PARTIAL SPECIFIC VOLUME AND WATER CONTENT OF INFLUENZA VIRUS*

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The knowledge of the density and the water content of virus particles in the native state is necessary for development of concepts of the structure and nature of the agents. Attempts have been made to approach the problem of wet density directly with several viruses (1-6) through study of the sedimentation velocity of the particulate agents in aqueous media of densities varied by the addition of certain solutes. In order to calculate the water content with the data obtained in this way, it is necessary, also, to know the partial specific volume of the particles.

The determinations of both wet density and partial specific volume have been associated with numerous difficulties. In studies on wet density, use of substances of low molecular weight, such as sodium chloride and sucrose, have provided data (1-5) indicating changes in the sedimentation properties of the viruses, vaccinia, and influenza, related to the concentrations of solute employed. In such experiments, an observed increase in density with increase in solute concentration appeared to be associated with withdrawal of water from the particles due to the high osmotic effect of the solute. Such effects were lessened, if not eliminated entirely, by use (5) of the high molecular weight bovine serum albumin, which, in the concentrations employed, exerted little osmotic effect, and no indication was seen of change in virus density related to its association with the virus. With this material, the relation of sedimentation rate to the density of the medium was linear, and the densities of influenza viruses A (PR8 strain) and B (Lee strain) and the swine influenza virus were determined. Estimations of partial specific volumes have thus far been made by weighings in the pycnometer, a technique of accuracy somewhat limited by the small amounts of some viruses available.

Recently additional studies have been made of the water content of these three types of influenza virus. One phase of the work was concerned with a method for determining partial specific volume other than

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by pycnometer weighings, namely, by measurement of the sedimentation properties of the influenza viruses in heavy water. This study was suggested by the finding, several years ago (7), that hemocyanin from the blood of *Helix pomatia* sedimented in saline solutions prepared with heavy water as in those prepared with ordinary water, and that, apparently, no change occurred in the partial specific volume of the protein related to the effects of heavy water. If this lack of effect likewise holds for influenza virus, it seemed possible to obtain an independent measure of partial specific volume from sedimentation velocity studies in heavy water. Another approach has been made, also, to the problem of water content by measurements on the volume of the mass of virus compressed in the ultracentrifuge. Such a method has been used in the investigation of the wet density of bacteria, but the technique employed¹ in the present work has the critical advantage of measurements of pellet volume while the centrifuge is running and while the virus mass is still compressed in a constant centrifugal field. The results of these studies² and those on the determination of partial specific volume with heavy water are described in this report.

Materials and Methods

The three types of influenza virus, influenza viruses A (PR8 strain) and B (Lee strain) and the swine influenza virus (strain 15), were the same as those employed in the previous studies (5) with bovine serum albumin. The viruses were cultured in the developing chick egg and sedimented from the chorio-allantoic fluid with the Sharples centrifuge (8). After the concentrate from the Sharples bowl, suspended in Ringer's solution, had been spun in an angle centrifuge at $1100 \times g$ for 10 minutes, the virus was again sedimented in the vacuum type ultracentrifuge at $20,000 \times g$ for 60 minutes. The pellets, resuspended in Ringer's solution, were spun at $1600 \times g$ for 10 minutes, and the virus was sedimented for the second time in the ultracentrifuge. The pellets were taken up in Ringer's solution and centrifuged, finally, at $700 \times g$ for 10 minutes. The resulting supernatant fluid containing the virus was employed for the experiments.

Studies on the volumes of sedimented virus were made with the air-driven analytical ultracentrifuge with a specially constructed lucite cell, which has been described in another report.¹ The technique involves sedimentation of the virus from suspensions of high virus concentration in a narrow column 2 cm. high and 0.0614 ml. in volume. Sedimentation of

¹ Sharp, D. G., *Biochim. et biophys. acta*, in press.

² The results reported here were described before the Division of Biological Chemistry at the meeting of the American Chemical Society at San Francisco, California, March 28 to April 1, 1949.

the virus and formation of the pellet at the bottom of the column can be followed while the centrifuge is running, and photographs can be made by a slight variation on the light absorption method of Svedberg (9) for observing sedimentation velocity. Estimations of pellet volume were made by measurements¹ of pellet height in the photographs with a traveling microscope calibrated in 0.001 mm. The volumes measured were of the order of 0.004 ml., and the accuracy was about ± 1 per cent.

The experiments³ with D₂O were made with solutions containing the kinds and amounts of salts⁴ present in Ringer's solution. In order to measure accurately the small amounts of salts necessary, small volumes of H₂O-Ringer solution were lyophilized, and the dry salts were redissolved in the appropriate volumes of D₂O or a mixture of D₂O and H₂O. The required amounts of virus were sedimented in the quantity ultracentrifuge, the collodion tubes employed were wiped dry with gauze, and the pellets were resuspended, easily, in the D₂O-Ringer solution. The 50 per cent point infectious units of swine influenza virus measured immediately, 3 days, and 13 days after suspension in D₂O-Ringer solution without H₂O, were $10^{-11.4}$, $10^{-11.4}$, and $10^{-11.3}$ gm. of N as compared with $10^{-14.1}$ gm. of N for the analogous unit of virus receiving the same treatment as that in D₂O, except that it was taken up in H₂O-Ringer solution. Similar findings with influenza viruses A and B indicated a like absence of effect of D₂O on the virus infectivity of these types of the virus.

Pycnometer measurements were made with a pycnometer of 2 ml. volume by the technique previously described (5). Nitrogen was determined by direct nesslerization. The speed of the analytical ultracentrifuge during the runs was controlled by a special governor.

EXPERIMENTAL

Sedimentation of Influenza Virus in Heavy Water—For these experiments, the sedimented virus was taken up in solutions of Ringer's salts in H₂O or D₂O or mixtures of the two fluids. The concentration of virus was about 2.0 mg. per ml. The results obtained in two series of studies with the swine influenza virus are shown in the circles and squares in Fig. 1. There are shown for comparison the findings in previous experiments (5) on the sedimentation of this virus in solutions of sucrose and solutions of bovine serum albumin. It is seen that, as in the case of serum albumin, the sedimentation rate appeared to be linear with the density of the suspending medium which was varied by different ratios of H₂O and D₂O. Similar

³ The D₂O was obtained with the permission of the Isotopes Branch of the United States Atomic Energy Commission, Oak Ridge, Tennessee.

⁴ The amounts of salts in 1 liter were NaCl 9.0 gm., KCl 0.42 gm., CaCl₂ (anhydrous) 0.24 gm., and NaHCO₃ 0.2 gm.

results were obtained with influenza viruses A and B and are compared with those with the swine influenza virus in Fig. 2. The values of the partial specific volumes of the three types of influenza virus, calculated on the assumption that partial specific volume was not altered by the effects of D_2O , are given, V_{D_2O} , in Table I.

Pycnometer Measurements—Estimations of partial specific volumes with the pycnometer were made with the three types of virus described above and previously⁵ and the values⁵ obtained, $V_{pyc.}$, are given in Table I.

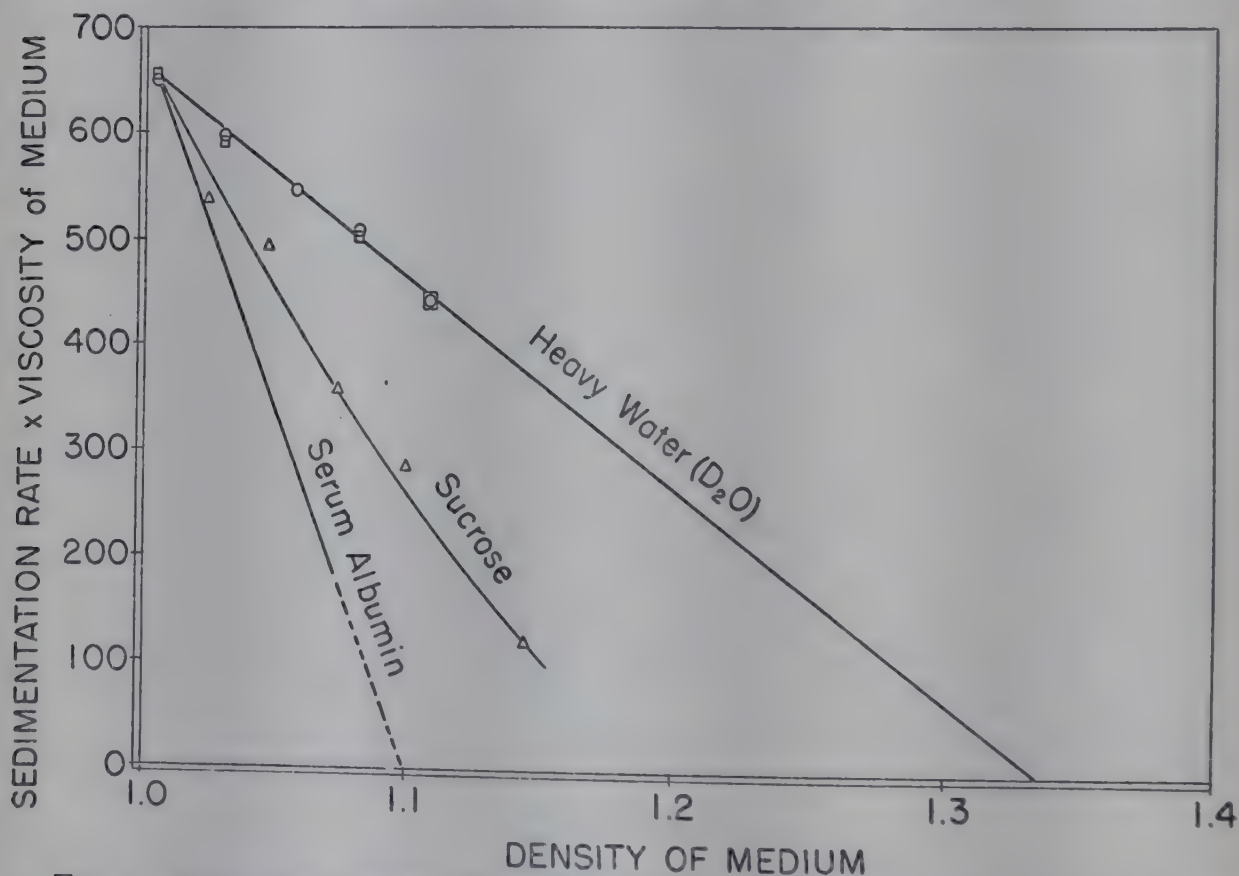


FIG. 1. Sedimentation rates of swine influenza virus in Ringer's solution of densities varied with different amounts of heavy water. \circ and \square represent data with two preparations. For comparison there are shown analogous data obtained in previous work (5) with swine influenza virus in Ringer's solution of densities varied with sucrose and with bovine serum albumin.

Measurements of Pellet Volume—The results of an experiment on sedimentation of influenza virus A are shown in Fig. 3. An appropriate volume of the stock purified virus was spun at $20,000 \times g$ for 60 minutes to

⁵ All three of these values were lower than those previously obtained (5) on the same strains of influenza viruses and the values obtained on influenza virus A (PRS strain) by Lauffer and Stanley (4). In contrast, the present preparations had the same sedimentation constants as those (5) observed before. It is not clear why this difference appears, but in the present work all measurements were made with freshly prepared virus, and as nearly as possible, under identical conditions.

sediment the virus. After the supernatant fluid was discarded, the virus pellets were resuspended in H₂O-Ringer solution to give a virus concentration of 16.4 mg. per ml. The volume of the special cell, 0.0614 ml., thus contained 1.0 mg. of virus. With this suspension, a series of four runs was made at different rotor speeds. The pellet volumes measured are shown in Fig. 3 plotted against time⁶ at a given constant rotor speed. The re-

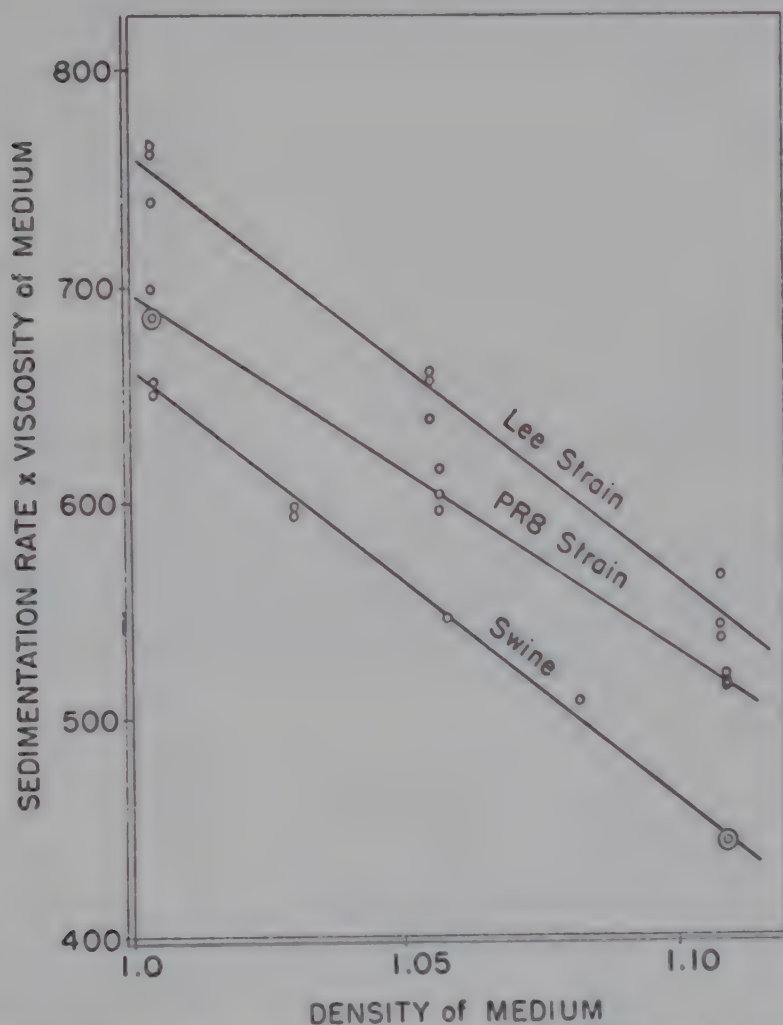


FIG. 2. Sedimentation rates of influenza viruses A (PR8 strain) and B (Lee strain) and swine influenza virus in Ringer's solution of densities varied with different amounts of heavy water.

sulting curves show the gradual compression of the virus to v_s as previously described¹ for the swine influenza virus.

The curves show also that as the rotor speed increased the rate of decrease in v_s decreased as well. This is shown again in Fig. 4 in which v_s for each of the speeds of Fig. 3 is plotted against ω^2 , the square of the

⁶ Pictures were taken as soon as the clearing of the turbid suspension above the pellet indicated that all of the virus was in the pellet. This accounts for the different volumes of the pellets at zero time in Fig. 3, though the amount of virus was constant in each run.

TABLE I
Partial Specific Volume and Water Content of Virus

Wet density of virus particle (ρ_v) determined with bovine serum albumin; partial specific volume of virus found with pycnometer ($V_{pyc.}$) and with D_2O (V_{D_2O}); density of virus pellet calculated from minimum pellet volume v_0 and $V_{pyc.}$ (ρ_{p^1}) and from minimum volume v_0 and V_{D_2O} (ρ_{p^2}); water content of virus particle, per cent of dry weight, calculated from ρ_v and $V_{pyc.}$ and ρ_v and V_{D_2O} ; and water content of the virus pellets, per cent of dry weight, from v_0 with $V_{pyc.}$ and with V_{D_2O} .

	Influenza virus		
	A	B	Swine
ρ_v	1.10	1.10	1.10
$V_{pyc.}$	0.75	0.76	0.76
V_{D_2O}	0.70	0.72	0.76
ρ_{p^1}	1.09	1.09	1.11
ρ_{p^2}	1.11	1.10	1.11
Particle water content* from ρ_v and $V_{pyc.}$	175	164	164
“ “ “ * “ “ “ V_{D_2O}	230	208	164
Pellet water content* from ρ_v and $V_{pyc.}$	205	203	147
“ “ “ * “ “ “ V_{D_2O}	210	207	147

* All measurements were made in Ringer's solution, about pH 7, and no distinction has been made between water content and solvent content of the virus particle or pellet. While relatively large uncertainties remain in measurements of this type, the distinction appears unnecessary.

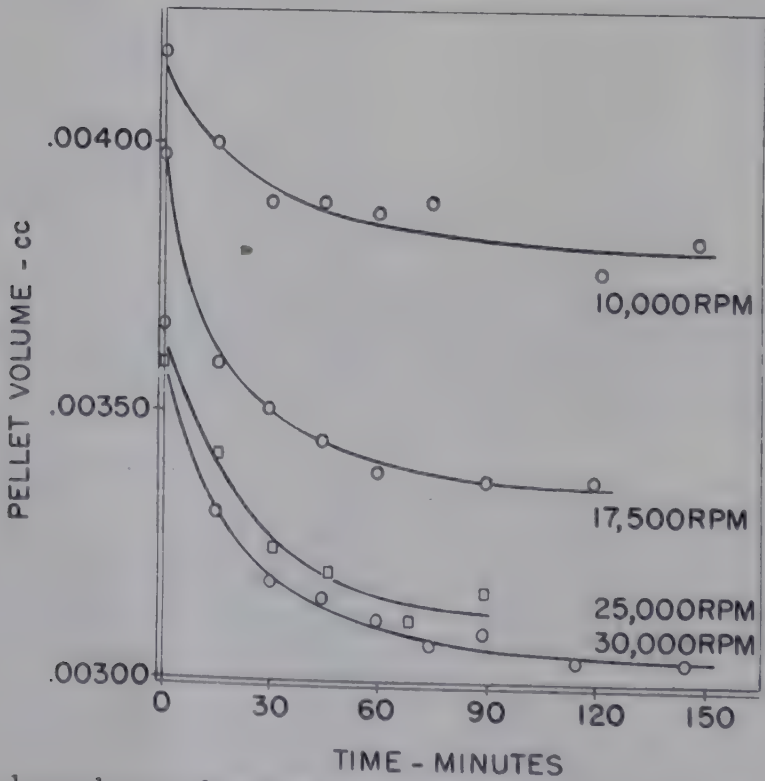


FIG. 3. The dependence of pellet volume on time at various angular velocities of the ultracentrifuge rotor.

angular velocity of the rotor. These points can sometimes be seen by inspection (Fig. 3), but in general they were obtained by extrapolation as follows:

$$v_t = v_s + \frac{k_1}{k_2 + t} \quad (1)$$

where v_t = the pellet volume at time t , v_s = the minimum volume approached at a given speed, and k_1 and k_2 are constants.

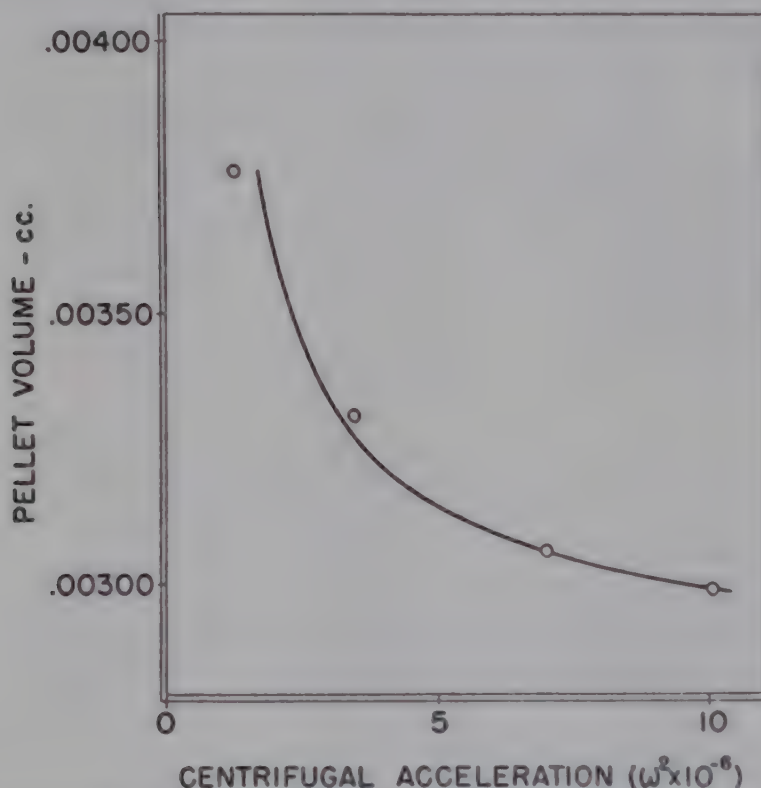


FIG. 4. The dependence of limiting pellet volume (v_0) on the angular velocity of the ultracentrifuge rotor squared (ω^2).

The values of the constants were chosen for best fit to the observed points, and the value of v_s was calculated for each speed. Each of the curves of Fig. 3 was calculated from the equation yielding the v_s . In Fig. 4 the curve was drawn from a similar equation.

$$v_s = v_0 + \frac{k_3}{k_4 + \omega^2} \quad (2)$$

in which v_0 = the pellet volume limit approached at high speed, ω = the angular velocity of the ultracentrifuge rotor, and k_3 and k_4 are constants.

The constants k_3 and k_4 were chosen for best fit with the observed points, and the curve of Fig. 4 was calculated from the equation, along with the value v_0 for the virus. It appears from Equation 2, taken together with the data described before¹ for the swine influenza virus, that the pellets

of these influenza viruses may indeed be elastic and that the pellets approach closely their minimum volumes in centrifugal fields of $76,000 \times g$, corresponding to 30,000 R.P.M. in the rotor of 7.55 cm. pellet radius. Data have been obtained similarly with influenza virus B and also with the swine virus.

The amount of virus present in the cell was determined by nitrogen measurements on the starting suspension, by use of the N factors previously reported (10), and checked by similar measurements on the total virus washed out of the cell after the run. The expected random error in these nitrogen measurements was considered to be comparable to or slightly larger than that of pellet volume determinations.

With the aid of the values of partial specific volume, $V_{\text{pyc.}}$, obtained by pycnometer measurements on all three virus suspensions as described above, 0.75 and 0.76 for influenza viruses A and B and 0.76 for the swine type, the data⁷ on pellet volumes can be expressed simply in terms of pellet density calculated as follows:

$$\rho_p = \frac{W_v + W_{\text{solv.}}}{v_0}$$

$$\rho_p = \frac{W_v + (v_0 - W_v V)\rho_0}{v_0} \quad (3)$$

where ρ_0 = the density of the solvent, Ringer's solution. Very small errors result in this case from taking $\rho_0 = 1$, whereupon the equation is simplified to

$$\rho_p = 1 + \frac{W_v(1 - v)}{v_0}$$

where ρ_p = pellet density; W_v = the weight of virus in the pellet; and $W_{\text{solv.}}$ = the weight of solvent (Ringer's solution) in the pellet; v_0 = pellet volume (equation (2)); and V = the partial specific volume of the virus.

The calculations yield pellet densities of 1.09 and 1.09 for influenza viruses A and B and 1.11 for the swine influenza virus (Table I) with use of the partial specific volumes, $V_{\text{pyc.}}$, obtained with the pycnometer. Similar calculations with partial specific volumes, $V_{\text{D}_2\text{O}}$, obtained with heavy water gave the analogous values 1.11, 1.10, and 1.11 for the pellet densities of the respective types of the virus.

⁷ From this point on, pellet volume will be taken to mean the limiting pellet volume, v_0 , approached as shown in equation (2) from curves like those of Fig. 2. ρ_p is then the density of this pellet.

DISCUSSION

Partial specific volume measurements have been made in the present work both with the pycnometer and by calculation from the results of sedimentation rate studies on three types of influenza virus in Ringer's solution of densities varied by the presence of different quantities of heavy water. The validity of the values obtained in the studies on sedimentation velocity are dependent on absence of effect of D_2O on those factors determining the sedimentation properties, principally the partial specific volume, of the suspended virus. That specific effects of the D_2O on the virus were exceedingly small, if they existed at all, was indicated by a number of findings. Measurements of the infectivity of preparations in D_2O showed no decline in virus activity within a period of 13 days, whereas all of the sedimentation studies were made within 48 hours. The dependence of sedimentation rate (corrected for viscosity) on the density of the suspending media was substantially linear with the three types of virus used. It is notable, also, that the data of Svedberg and Eriksson-Quensel (7) showed no effect of D_2O on the partial specific volume of the hemocyanin of *Helix pomatia*.

In Fig. 1 there are shown the sedimentation velocity data for swine influenza virus in D_2O -Ringer solution, and from these data, V_{D_2O} was calculated as described. As far as can now be determined, this procedure should be valid, but agreement with $V_{pyc.}$ was obtained only in the case of swine virus in the present work, while lower values were obtained with influenza viruses A and B. Although it is still possible that minor changes occurred in particle structure induced by the D^+ ion, the top curve of Fig. 1 shows apparently complete replacement of associated H_2O with D_2O . At the other extreme is the serum albumin curve showing little or no penetration of albumin molecules into the water volume associated with the virus. The sucrose curve lies between these extremes. It would seem improbable that water to the extent of about 200 per cent of the dry weight, Table I, lies in a layer outside the virus particle. Serum albumin would be expected to penetrate most of such a layer about this large virus particle, except for small contact region of exclusion rising from finite size of the albumin molecules (11). The water must then lie within the virus structure, inaccessible to albumin molecules but able to dissolve some sucrose. Either the water is not all available to sucrose or else space limitations within the virus structure must limit the amount of sucrose that can enter; otherwise sucrose and D_2O should give the same curve of viscosity times sedimentation constant *versus* density in Fig. 1.

The use of D_2O should be particularly useful for animal viruses obtainable only in limited quantity, since determinations of partial specific volume can be made with the method on about one-tenth of the amount of

virus needed for pycnometer measurements of the same degree of accuracy. It is possible also to make these measurements with D_2O on relatively impure virus samples if the impurities do not sediment at the same rate as the virus boundary. In fact, if further work shows the values of V_{D_2O} so obtained to be the true partial specific volume of the pure material, it will be possible, by a combination of this and the serum albumin technique for getting wet density, to obtain particle size, weight, and water content of spherical viruses from the ultracentrifuge alone, and by methods less sensitive to impurities in the virus preparations than by other methods currently used.

The virus pellets sedimented in the ultracentrifuge in the present experiments gradually compress to a minimum volume at a given constant rotor speed (Fig. 3). These minimum volumes plotted for a series of speeds against the square of the rotor speed vary in a manner (Fig. 4) suggesting that the pellets consist of compressible, elastic material of definite minimum volume closely approached in a centrifugal field of $76,000 \times g$ (30,000 R.P.M.). As seen in Fig. 4, an excellent fit with the data is obtained with the curve of equation (2) which embodies this concept and from which the apparently irreducible volume has been calculated for the three types of influenza virus used. A direct demonstration of the elasticity of the virus particles was seen in the previously described¹ experiments with swine influenza virus. In this case the pellet volume observed at $76,000 \times g$ increased on deceleration to $4750 \times g$ almost to the volume reached by centrifugation only at $4750 \times g$.

The significance of the minimum pellet volumes can, of course, not be established definitely until the pellet volumes have been measured under a variety of conditions such as, for example, those involving pH and salt and virus concentrations; nevertheless, it is notable that the densities of the virus pellets (Table I) calculated on the basis of minimum pellet volumes are remarkably close to the values for wet density of the virus particles obtained in previous work (5) by sedimentation through bovine serum albumin. Such agreement might be explained by supposing the virus *particles* to be subject to deformation during packing but not to significant volume change. *Pellet volume* may be decreasing in these experiments only to the point at which the individual particles are deformed to occupy all of the space and water has been excluded from the interstices between them. Table I shows, in addition to the comparative densities, the water content (see the foot-note of Table I) of the pellets. Both density and water content were calculated by using the partial specific volume of the viruses measured with the pycnometer. Water values were calculated also through use of these partial specific volumes and the data published previously on wet density given in the first line of Table I. Still further

values were obtained with the partial specific volumes obtained with sedimentation experiments in D_2O . All of the four sets of values of water content are higher than those previously reported (5) because of the differences in partial specific volume discussed above,⁵ though the results obtained with the three types of virus are very much alike in the present work. Beyond showing similarity in water content of pellet and particle, these values indicate an amount of water several fold greater than that usually considered *bound* in biological materials. This excess water is probably no more bound in the virus than is most of the water bound in a plant or animal cell, but it seems to be held by forces considerably greater than the relatively mild⁸ ones applied to compress the pellets in these ultracentrifuge experiments. It is possible that the minimum pellet volume approached, Fig. 4, indicates a minimum water content characteristic of the structure of the virus particle. The force required to effect compression may be a measure of the mechanical strength of the compression members within the virus structure or, possibly, may be a measure of the osmotic pressure exerted across a semipermeable membrane about the virus particle.

Gelatin compressed in a screw press (12) has been shown to lose water with increased pressure, and the relation of compression pressure to gelatin volume is not unlike that shown in Fig. 3 for virus pellets in the ultracentrifuge. There is, however, no plateau or discontinuity shown with gelatin in the region of 200 per cent hydration but only the plateau associated with bound water which is revealed at a much lower level and much greater pressures. It is probable that sufficient pressure on virus pellets would overwhelm the forces responsible for the minimum volume plateau seen in the present experiments, causing rupture of the virus particles and release of all water except that held to their constituent materials by the chemical bonds associated with bound water.

SUMMARY

Purified influenza viruses A (PR8 strain) and B (Lee strain) and the swine influenza virus have been studied to determine the water content of the virus in aqueous suspension. In one type of experiment the volume of sediment from a suspension containing a known weight of virus has been measured in the ultracentrifuge. The pellets of such sediment show volume elasticity through reversible changes with the amount of ultracentrifugal

⁸ For a pellet 0.1 cm. high and density 1.1 in a centrifugal field of $76,000 \times g$, the pressure exerted on the bottom of the cell would be $76,000 \times 0.1 (1.1 - 1.0) = 760$ gm. per sq. cm., if the pellet were in water. The pressure at the bottom of the cell thus would be about 0.7 atmosphere, while the particles at the top of the pellet would be subjected to smaller compressing forces. The force gradient existing radially in the pellet might be studied further as a means of learning the minimum force needed to deform the virus particle.

force applied. The pellets can be compressed at increasing speed, but the volume approaches a limiting value which has been calculated from data on all three viruses. This limiting pellet volume has been used to calculate its density and water content. Values so obtained are in quite good agreement with those measured for the sedimenting virus particles by independent methods. They both show water associated with the virus to the extent of about 200 per cent of the dry weight of the latter.

Possibly the greatest uncertainty in these measurements lies in the values of partial specific volume used. Although the above data are based on values from the pycnometer, an alternative method is described for measuring partial specific volume which should be particularly useful for materials available only in the very small quantities. It involves comparison of the sedimentation rates of the virus particles through D_2O and H_2O in the analytical ultracentrifuge.

It is clear that much more water is associated with the influenza viruses than the amount usually ascribed to chemical binding of their constituent materials. This suggests a loose but strong structure containing water, possibly surrounded by a membrane.

BIBLIOGRAPHY

1. Schlesinger, M., *Z. Hyg. u. Infektionskr.*, **114**, 161 (1932-33).
2. Elford, W. J., and Andrewes, C. H., *Brit. J. Exp. Path.*, **17**, 422 (1936).
3. Smadel, J. E., Pickels, E. G., and Shedlovsky, T., *J. Exp. Med.*, **68**, 607 (1938).
4. Lauffer, M. A., and Stanley, W. M., *J. Exp. Med.*, **80**, 531 (1944).
5. Sharp, D. G., Taylor, A. R., McLean, I. W., Jr., Beard, D., and Beard, J. W., *J. Biol. Chem.*, **159**, 29 (1945).
6. Lauffer, M. A., *J. Biol. Chem.*, **174**, 481 (1948).
7. Svedberg, T., and Eriksson-Quensel, I.-B., *Nature*, **137**, 400 (1936).
8. Taylor, A. R., Sharp, D. G., McLean, I. W., Jr., Beard, D., and Beard, J. W., *J. Immunol.*, **50**, 291 (1945).
9. Svedberg, T., and Pedersen, K. O., *The Ultracentrifuge*, Oxford (1940).
10. Taylor, A. R., *J. Biol. Chem.*, **153**, 675 (1944).
11. Schachman, H. K., and Lauffer, M. A., *J. Am. Chem. Soc.*, **71**, 536 (1949).
12. Lloyd, D. J., and Moran, T., *Proc. Roy. Soc. London. Series A*, **147**, 382 (1934).

THE EFFECT OF ADDED CARRIER ON THE DISTRIBUTION AND EXCRETION OF SOLUBLE Be^7 *

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The experiments described below were carried out to determine whether the distribution and excretion of a soluble beryllium compound, given intravenously, would be altered by the size of the dose, also, to determine the effect of age and changes in urinary pH on the distribution and excretion. Studies on the metabolism of beryllium following intramuscular injection with carrier-free Be^7 have been reported by Crowley *et al.* (1). Their findings in general conformed with ours, except that the mobilization of the isotope from the site of injection was slower than would be expected from our results; however, the differences in the route of administration and the dosage of the beryllium do not allow strict comparisons.

EXPERIMENTAL

Methods

Rabbits and rats were employed in these experiments. The preparation of Be^7 for injection was performed without adding beryllium salts (2); thus (except for any unavoidable contamination that might have occurred) only the beryllium injected was isotopic and, consequently, the dosage in animals receiving isotope only was small, approximately 9.3×10^{-11} gm. of beryllium per kilo for rats and 4.4×10^{-11} gm. of beryllium per kilo for rabbits. For a large but sublethal dose, beryllium sulfate was added to the isotope, 1.5×10^{-4} gm. of beryllium per kilo for rats and 5.0×10^{-5} gm. of beryllium per kilo for rabbits. The designations, isotope only and isotope plus carrier, will be employed to indicate these two types of dosage. The urine of the rabbits was changed from its usual alkaline reaction to an acid one (pH 5.3 to 6.0) by feeding the animals oats and bread. All data are based on the total amount of isotope recovered from the animal. All solutions were injected intravenously.

* This paper is based on work performed under contract with the United States Atomic Energy Commission at the Atomic Energy Project of The University of Rochester, Rochester, New York.

Results

The *urinary excretion* of beryllium in *rats* is given in Table I. It will be noted that age exerted no significant influence on the excretion of beryllium if carrier was used and little influence if only isotope was used. In all cases the greatest excretion occurred during the first 24 hours, and it was only during this period that a significant effect of added carrier was observed. After the first 24 hours, the daily excretion of Be⁷ was about the same in animals receiving carrier and those not receiving carrier, and

TABLE I
Urinary Excretion of Be⁷ in Rats during First 24 Hours

Average of two rats in each group.

	Age of animals	
	120 days	35 days
	<i>per cent</i>	<i>per cent</i>
Isotope only.....	38.8	31.1
“ + carrier.....	24.2	20.5

TABLE II
Urinary Excretion of Be⁷ for Rabbits

Average of six animals in isotope only group and three animals in isotope plus carrier group.

	6 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.	120 hrs.	144 hrs.	168 hrs.	Total hrs.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Isotope only.....	27.3	1.5	0.7	0.7	0.6	0.6	0.5	0.5	32.4
“ + carrier.....	12.2	1.8	0.9	1.3	0.8	0.5	0.7	0.5	18.7

there was a gradual decrease in excretion each day from an average of 1.2 per cent of the total dose on the 2nd day to 0.3 per cent on the 7th day.

The period of greatest *urinary excretion* of beryllium in *rabbits* was during the first 6 hours after injection, and variations induced by experimental procedures were observed only during this period. Age did not affect significantly the excretion of beryllium, since the young rabbits excreted an average of 6 per cent less than the adults. When beryllium sulfate was added to the isotope, the per cent of isotope excreted during the first 6 hours was approximately one-half that of the animal receiving only isotope. The data are given in Table II. During the 1st hour after intravenous administration of carrier-free isotope, the rabbits with acid urine excreted more of the dose than the animals with an alkaline urine (see Fig. 1).

These differences in excretion, apparently resulting from the changes in the reaction of the urine, were not observed in animals receiving carrier in addition to the isotope.

Fecal Excretion of Beryllium—The average total fecal excretion of Be^7 over a 7 day period was 9.8 per cent of the dose in the rat and 2.3 per cent in the rabbit. In rabbits the per cent excreted daily increased gradually, reaching a peak on the 4th day; thereafter it decreased gradually. No significant difference was observed in animals receiving carrier and those

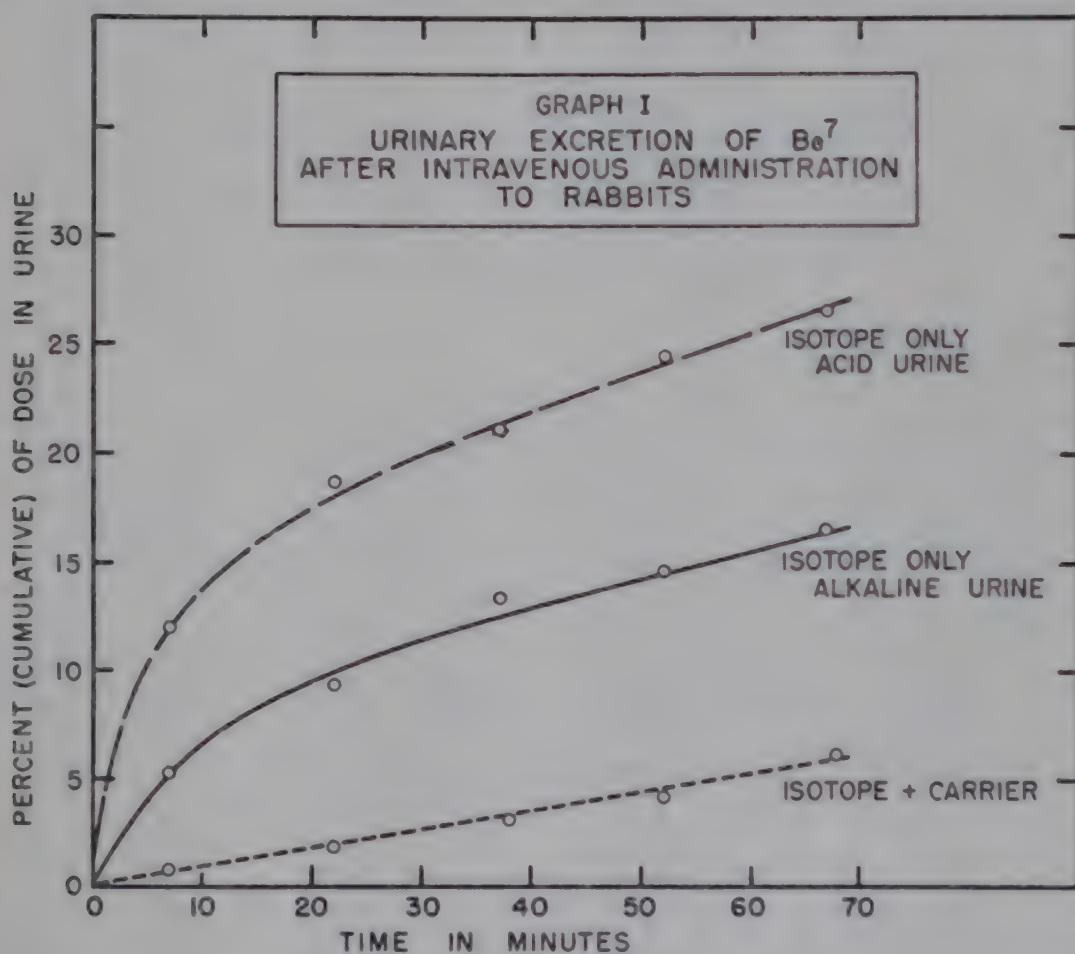


FIG. 1

receiving isotope only. In rats the total per cent of isotope excreted over a 7 day period was about twice as great in animals which received isotope plus carrier as those which received only the isotope. In all rats the per cent excreted on the 1st day was greatest (3 to 5 per cent) and was about the same in the animals that received isotope plus carrier as in those which received only isotope; however, during the subsequent 6 days the animals that received carrier excreted a higher per cent daily, and it is this which accounted for the differences in the total. The data are given in Table III.

To arrive at some idea of the portion of the gastrointestinal tract in

which fecal excretion of beryllium takes place, the stomach, small intestine, contents of the small intestine, large intestine, and contents of the large intestine of rats were examined separately. Significant amounts of beryllium were not found in any part of the gastrointestinal tract of animals receiving isotope only; that is, the per cent of isotope per gm. of tissue was not greater than the "soft carcass."¹

On the other hand, in rats administered carrier with the isotope, the wall of the small intestine uniformly contained significant amounts of the isotope (about 1.4 per cent of the dose) (see Table IV). In the rabbit, de-

TABLE III
Daily Fecal Excretion of Be⁷ in Rabbits and Rats

	1 day	2 days	3 days	4 days	5 days	6 days	7 days	Total
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Rabbits	0.1	0.3	0.3	0.5	0.3	0.3	0.2	2.0
Rats, isotope + carrier	4.2	1.6	2.0	1.2	1.1	1.0	0.7	11.8
Rats, isotope only . .	3.5	0.8	0.4	0.3	0.2	0.2	0.2	5.6

TABLE IV
Distribution of Be⁷ in Gastrointestinal Tract in Rats Receiving Isotope Plus Carrier

Rat No.	Stomach and contents	Small intestine	Contents of small intestine	Large intestine	Contents of large intestine	Soft carcass	Per cent of dose in small intestine
	<i>per cent per gm.</i>	<i>per cent per gm.</i>	<i>per cent per gm.</i>	<i>per cent per gm.</i>	<i>per cent per gm.</i>	<i>per cent per gm.</i>	
90	0.02	0.3	0.05	0.1	0.04	0.05	1.4
91	0.02	0.2	0.1	0.2	0.05	0.05	1.0
102	0.06	0.5	0.1	0.1	0.03	0.05	1.8
103	0.02	0.5	0.04	0.2	0.05	0.05	1.5

tectable amounts of Be⁷ were present in all parts of the gastrointestinal tract, but in counts per gm. of tissue all, except the cecum and large intestine, were less than the soft carcass; the counts per gm. of tissue of the cecum and large intestine were roughly the same as those of the soft carcass. The gall-bladder and its contents were analyzed separately and contained about the same amount of Be⁷ in counts per gm. as the soft carcass.

¹ A large number of organs (lungs, heart, brain, etc.) and tissues (muscle, fat, skin, etc.) contain small numbers of counts but, when added together, account for 5 to 10 per cent of the dose. Soft carcass is used to represent this small but uniform distribution, and it is thought that, if the counts per gm. of tissue are not greater than those found in the soft carcass, they are not significant.

Clearance of Be^7 from Blood—This experiment was performed on rabbits. The rate of clearance of the isotope from the blood was much slower when carrier was injected with the isotope than when the isotope

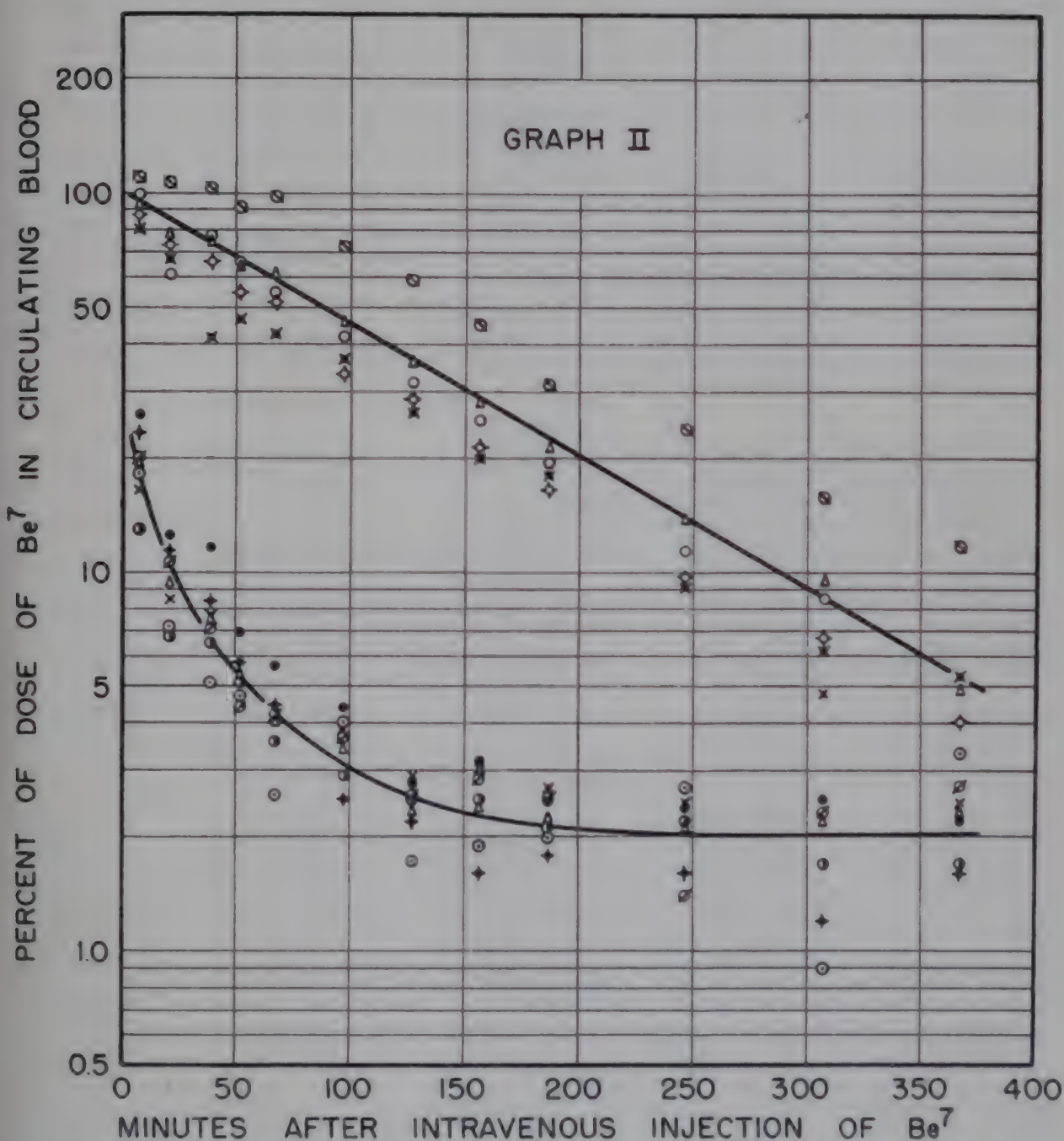


FIG. 2. The blood clearance of Be^7 in rabbits. The upper line represents the blood clearance in animals receiving isotope plus carrier and the lower line isotope only. The symbols indicate data on individual animals.

alone was injected. When the isotope only was injected, approximately 80 per cent of the dose was cleared from the blood within 7 minutes, and after 2 hours the amount of isotope in the blood remained fairly constant, which would indicate that the isotope was at equilibrium with the extra-vascular tissues and fluids. The results are given in Fig. 2. It will be

noted that the slope of the curve is fairly constant for individual animals, but that there is considerable variation in the per cent of isotope present in the blood at any given time. This variation is probably a result of differences in blood volume of the individual animals and errors in the total recovery of the isotope from the animal at the time of dissection. All of the blood samples examined between the 1st and 7th day contained counts, and, although these counts represented only a small amount of the total dose, they indicated that small amounts of beryllium were present in the circulating blood at all times. In the rabbits which received carrier in addition to isotope, this amounted to 3 to 6 $\mu\text{gm.}$ per gm. of blood. The percentage of isotope present in the blood of rabbits receiving only isotope was about the same as in those also receiving carrier; however, the amount of beryllium was much greater in animals receiving carrier.

TABLE V

Distribution of Be⁷ in Organs and Excreta of Rats and Rabbits
Average of four animals in each group.

		Skeleton	Liver	Spleen	Kidney	Urine	Feces	Carcass
		per cent	per cent	per cent	per cent	per cent	per cent	per cent
Rats	Isotope + carrier.....	35.2	14.0	2.8	0.6	26.1	12.6	5.9
	" only.....	48.3	0.9	0.1	0.7	37.6	5.5	5.6
Rabbits	" + carrier.....	51.2	18.7	0.5	0.5	17.9	1.6	9.6
	" only.....	53.0	5.7	0.1	0.7	32.9	2.3	5.3

The *organs* of the *rat* which contained most of the beryllium that had not been excreted 7 days after intravenous injection were the skeleton, liver, spleen, and kidneys. The age of the animals made no significant difference in amount deposited in the different organs. The presence or absence of added carrier made considerable differences. In those animals receiving carrier, the liver and spleen contained appreciable amounts of beryllium, whereas in those animals receiving only isotope, the liver and spleen contained small amounts of isotope (approximately the same amount per gm. of tissue as found in the soft carcass), and a higher per cent was found in the skeleton. The distribution among the organs of the rabbit followed in general much the same pattern as in the rat; however, the differences (spleen and liver content) resulting from the presence of carrier were not so marked as in the rat. The data are given in Table V.

In the rabbits the marrow of the femur and tibia was removed from the bone and analyzed separately. Considerable variability in the per cent of isotope in these marrow samples was encountered; however, the marrow of animals receiving isotope plus carrier uniformly contained more counts

than those receiving only isotope, and the latter were in most cases about the same as the soft carcass. The results of these examinations are given in Table VI.

TABLE VI

Distribution of Be⁷ in Skeleton and Bone Marrow

Average of four rabbits in each group.

	Tibial marrow	Femoral marrow	Tibial epiphy- sis	Femoral epiphy- sis	Tibial diaphy- sis	Femoral diaphy- sis	Total skele- ton
	per cent per gm.	per cent per gm.	per cent per gm.	per cent per gm.	per cent per gm.	per cent per gm.	per cent per gm.
Isotope only	0.02	0.05	0.4	0.2	0.2	0.3	0.2
" + carrier	0.1	0.2	0.4	0.4	0.2	0.2	0.2

DISCUSSION

Many of the differences observed in the distribution and excretion of the Be⁷ when isotope alone and when isotope plus carrier were administered intravenously may be explained on the low solubility of beryllium salts at the pH of body fluids (3). The rather rapid excretion of the isotope in the urine during the period when the blood concentration is high would indicate that some of the beryllium is diffusible at this time. The extremely rapid clearance of Be⁷ from the blood when isotope alone was injected probably is a result of a rapid uptake by the bone, since such a small amount of beryllium present is probably soluble in the body fluids. The slower blood clearance when isotope plus carrier was injected seems to indicate that some of the beryllium is insoluble and that its removal from the circulating blood was brought about in part by the reticulo-endothelial system, since the liver, spleen, and bone marrow, in addition to bone and urine, contained the isotope (4). The amount of beryllium excreted in the urine after isotope plus carrier was administered is much greater than when isotope alone was given. This difference may be a result of more rapid mobilization of the element from the liver, spleen, and bone marrow and a slower mobilization from the bone, since bone is the only tissue containing large amounts of beryllium when only isotope is injected. The same explanation may hold for the fecal excretion of the beryllium; however, this does not account for the marked species difference.

SUMMARY

1. When isotope only was injected intravenously, most of the isotope was excreted in the urine and deposited in bone. When carrier plus iso-

tope was injected, beryllium was found in the liver, spleen, and bone marrow, in addition to the urine and bone.

2. The clearance of beryllium from the blood was much faster when isotope only was injected than when isotope plus carrier was injected.

3. Significant amounts of beryllium were excreted in the feces of rats when beryllium plus isotope was injected.

The authors gratefully acknowledge the technical assistance of Geraldine Bonner, Arnold Sparks, and George Kosel, and the advice of J. F. Bonner, Jr., on counting techniques.

BIBLIOGRAPHY

1. Crowley, J. F., Hamilton, J. G., and Scott, K. G., *J. Biol. Chem.*, **177**, 975 (1949).
2. Bonner, J. F., Jr., United States Atomic Energy Commission, Document UR-56 (1949).
3. Prytz, M., *Z. anorg. Chem.*, **179**, 355 (1929).
4. Dobson, E. L., Gofman, J. W., Jones, H. B., Kelly, L. S., and Walker, L. A., *J. Lab. and Clin. Med.*, **34**, 305 (1949).

STUDIES IN STEROID METABOLISM

VII. IDENTIFICATION AND CHARACTERIZATION OF ADDITIONAL KETOSTEROIDS ISOLATED FROM URINE OF HEALTHY AND DISEASED PERSONS*

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The identification and characterization of forty-two urinary ketosteroids were described in the second paper of this series (1). This paper presents an extension of that study in which the isolation of seven new steroids from human urine is described; in addition, four compounds, Compounds A5, A26, A31, and A32, which were partly characterized previously, have now been identified. Ten of these substances are listed in Table I; the compound number indicates the position of the compound in the sequence of its elution in the chromatographic separation with reference to the steroids listed in Table I of Paper II. Two compounds (pregnanediol-3 α ,20 α -one-11 and etiocholenediol-3 α ,17 β -one-11) have not been numbered, since they occur in the α -non-ketonic alcoholic fractions (2) which will be discussed in a subsequent publication.

Methods

Since the methods for the isolation, estimation, and characterization of urinary ketosteroids already have been described (1, 2), only certain special procedures which bear directly on the isolation of certain of the compounds will be discussed here. Two ketonic compounds (pregnanediol-3 α ,20 α -one-11 and etiocholenediol-3 α ,17 β -one-11) have been isolated from the *non-ketonic*, alcoholic fractions because the carbonyl groups of these substances located on C₁₁ do not react with Girard's reagent.

The separation of ketosteroids into α and β subfractions by means of digitonin is a satisfactory and useful procedure (2), in spite of the fact that it is not completely specific (3). Δ^2 (or 3)-Allopregnenone-20 (Compound

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TABLE I
Urinary Ketosteroids

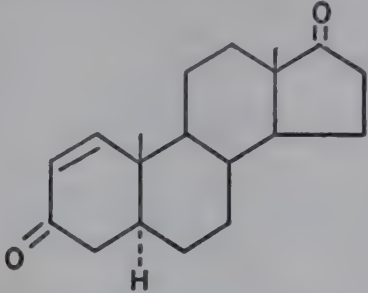
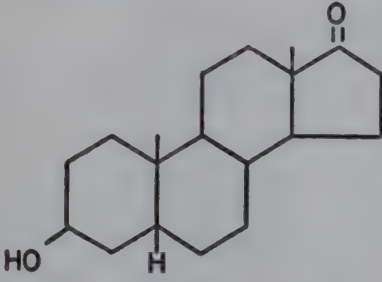
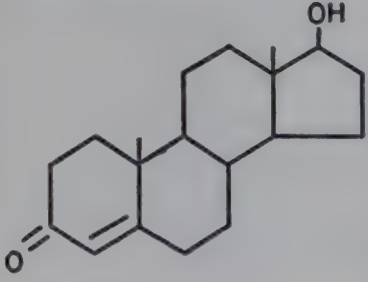
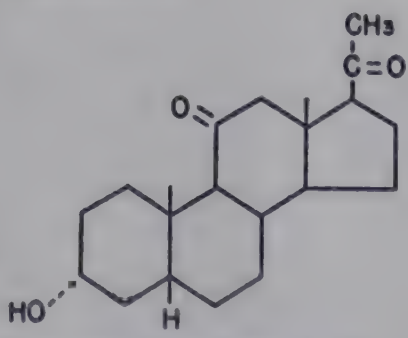
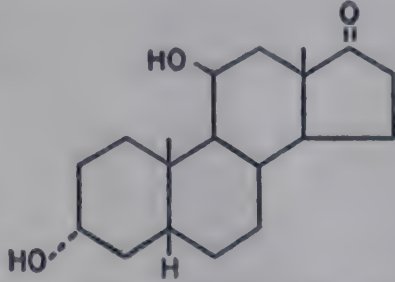
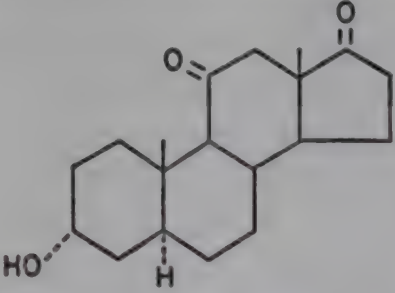
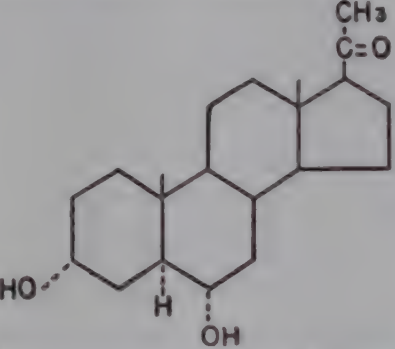
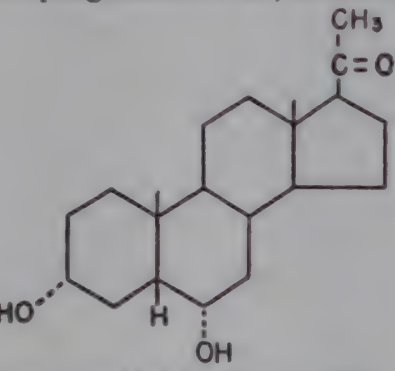
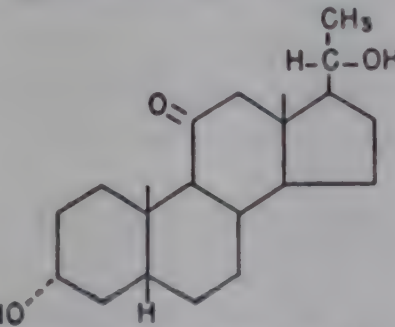
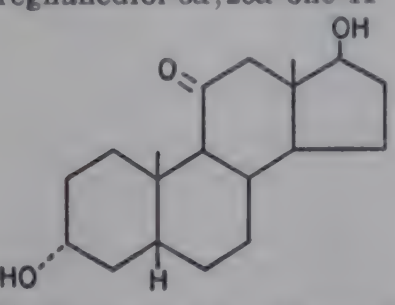
Compound No.	Substance	Empirical formula	Identifying characteristics
A13a	 Δ^1 -Androstenedione-3,17	$C_{19}H_{26}O_2$	M.p. 138–142°; $[\alpha]_D = +122^\circ$; absorption maximum at 232 $m\mu$, $\log \epsilon = 4.10$
A19a	 Etiocholanol-3 β -one-17	$C_{19}H_{30}O_2$	M.p. 154–155°; an insoluble diglucuronide; acetate, m.p. 151–155°
A24a	 Testosterone	$C_{19}H_{28}O_2$	M.p. 143–151°; absorption maximum at 239 $m\mu$
A23	 Pregnanol-3 α -dione-11,20	$C_{21}H_{32}O_3$	M.p. 174.5–175°; $[\alpha]_D = +111^\circ$; oxidized to pregnanetrione-3,11,20, m.p. 158–160°; $[\alpha]_D = +125^\circ$
A27a	 Etiocholanediol-3 α ,11 β -one-17	$C_{19}H_{30}O_3$	M.p. 236–237°

TABLE I—Concluded

Compound No.	Substance	Empirical formula	Identifying characteristics
A28a	 <p>Androstanol-3α-dione-11,17</p>	C ₁₉ H ₂₈ O ₃	M.p. 153.5–155°; [α] _D = +127°; acetate, m.p. 183.5–184.5°; [α] _D = +113°
A31	 <p>Allopregnanediol-3α,6α-one-20</p>	C ₂₁ H ₃₄ O ₃	M.p. 195–196°; [α] _D = +105°; diacetate, m.p. 155–156°; oxime, m.p. 288–291°
A32	 <p>Pregnanediol-3α,6α-one-20</p>	C ₂₁ H ₃₄ O ₃	M.p. 192–194°; [α] _D = +66.3°; low melting form from benzene, m.p. 95–105°; diacetate, m.p. 128–129°; dinitrophenylhydrazone, m.p. 234–238°
	 <p>Pregnanediol-3α,20α-one-11</p>	C ₂₁ H ₃₄ O ₃	M.p. 214–217°; diacetate, m.p. 231–233°; [α] _D = +56.7°
	 <p>Etiocholanediol-3α,17β-one-11</p>	C ₁₉ H ₃₀ O ₃	M.p. 262–262.5°; diacetate, m.p. 182–183.5°; [α] _D = +46.9°

B1) has been listed (1) among the β -ketosteroids because it was precipitated with digitonin, although it does not contain a 3β -hydroxyl group. We have encountered two 3β -hydroxy steroids, dehydroisoandrosterone (Compound A5) and etiocholanol- 3β -one-17 (Compound A19a), in the fraction not precipitated by digitonin. When dehydroisoandrosterone is present in very large amounts, small quantities fail to precipitate with digitonin and appear in the α -ketonic fraction. Until now etiocholanol- 3β -one-17 (Compound A19a) has been found only in the α fraction. Although the 3β -hydroxy steroids are occasionally found in the α fraction, the 3α -hydroxy steroids rarely, if ever, are detected in the β fraction. These limitations of the digitonin separation should be borne in mind.

The separation of isomeric pairs of ketosteroids by chromatography was readily accomplished on alumina (see (2) Table IV), with pairs of saturated diastereoisomers such as allopregnanolone and pregnanolone, and androsterone and etiocholanolone. However, great difficulty was encountered in the separation of 11-ketoetiocholanolone (Compound A28) and 11-ketoandrosterone (Compound A28a). Only after several chromatograms was it possible to separate Compound A28a from its isomer. When two compounds differ by only one double bond, only partial separation was achieved by chromatography. The following pairs were difficult to separate: $\Delta^{3,5}$ -androstadienone-17 (Compound A2) and Δ^2 (or 3)-androstenone-17 (Compound A3); androsterone (Compound A20) and Δ^9 -androstenol- 3α -one-17 (Compound A21); Δ^9 -etiocholenol- 3α -one-17 (Compound A23) and etiocholanol- 3α -one-17 (Compound A24); isoandrosterone (Compound B3) and dehydroisoandrosterone (Compound B4).

Constitution and Order of Elution

Correlations between chemical constitution and relative order of elution during chromatography have been discussed in a previous publication (1). The following generalizations can be made on the basis of our present knowledge.

1. C-21 pregnane derivatives are eluted prior to their corresponding C-19 androstane derivatives.

2. Compounds with Ring A-B trans juncture (androstane and allopregnane) are eluted before their corresponding isomers with Rings A-B cis (etiocholane and pregnane). One exception to this rule has been noted: etiocholanol- 3β -one-17 is eluted from alumina before androstanol- 3α -one-17. This inversion in the expected sequence of elution may be a special case because these substances also differ in their configuration at C₃. The configuration of the 3-hydroxyl group seems to be more influential in determining the chromatographic sequence than does the steric juncture of Rings A and B.

3. Saturated compounds are eluted before their unsaturated analogues except in the case of etiocholanolone (Compound A24), which is eluted after $\Delta^{9,11}$ -etiocholenolone (Compound A23). Δ^1 -Androstenedione-3,17 (Compound A13a), as expected, is eluted after its saturated analogue, androstenedione-3,17 (Compound A12). The unsaturated Δ^1 -compound (Compound A13a) precedes its Δ^4 isomer (Compound A14) in the sequence of elution.

4. The diketomonohydroxy compounds are eluted before their corresponding dihydroxymonoketo compounds with the important exception that the dihydroxymonoketones having one hydroxyl group on C₁₁ are eluted before those diketomonohydroxy compounds with one ketonic group on C₁₁. Thus, 11-hydroxyandrosterone (Compound A27) and 11-hydroxy-etiocholanolone (Compound A27a) are eluted *before* their corresponding C₁₁ carbonyl derivatives, 11-ketoandrosterone (Compound A28a) and 11-ketoetiocholanolone (Compound A28). The remaining generalizations in Paper II (1) remain unaltered.

These rules do not necessarily hold for adsorbents other than alumina. For example, 11-ketoandrosterone (Compound A28a) and 11-ketoetiocholanolone (Compound A28) are eluted in the expected order from alumina (Compound A28 < Compound A28a); however, when magnesium silicate-Celite is used as the adsorbent, the order of elution is inverted. Another instance of such inversion has been found with the two β steroids, dehydroisoandrosterone (Compound B4) and isoandrosterone (Compound B3). Isoandrosterone is eluted from alumina before dehydroisoandrosterone but on magnesium silicate-Celite the order is reversed. Both on alumina and on magnesium silicate-Celite chromatograms the isomeric pair, allo-pregnanediol-3 α -6 α -one-20 (Compound A31) and pregnanediol-3 α ,6 α -one-20 (Compound A32), is eluted in the usual order, the *trans* compound (allopregnane) being eluted before the *cis* (pregnane) compound.

Infra-Red Spectrometry

Infra-red spectrometry, when employed in combination with other analytical procedures, especially chromatographic analysis, has already been shown to be of the greatest value for the detection and identification of urinary steroids (4-8). Every steroid has unique absorption in the fingerprint region (1200 to 800 cm.⁻¹) and it is easily possible to recognize a known compound or binary mixtures of known compounds in a chromatographic eluate. A new compound can also be detected from its infra-red spectrum. The spectra of more than 500 steroids, including many substances likely to be metabolites, have been determined. It has been possible to detect many steroids not isolated previously from urine by comparison with the reference spectra. Compounds which have been detected by

infra-red analysis in fractions from human urine for the first time are Δ^1 -androstenedione-3,17 (Compound A13a), etiocholanol-3 β -one-17 (Compound A19a), testosterone (Compound A24a), etiocholanediol-3 α -11 β -one-17 (Compound A27a), and 11-ketoetiocholanediol-3 α ,17 β . Some compounds were specifically prepared for the purpose of recording their spectra in order to compare the known spectrum with that of a chromatographic eluate. It was possible in this way to establish the presence of 11-ketoandrosterone in urine. Search for other 11-ketosteroids was undertaken because the carbonyl group at C₁₁ exhibits an absorption band at 1716 to 1710 cm.⁻¹ and this is characteristic for this position (6). 11-Ketopregnanediol and 11-ketoetiocholanediol were detected by this technique and, following isolation, their structure was established by chemical means. Spectroscopic examination of two compounds which previously had been only partially characterized (1) because of the small amount of material has resulted in the identification of Compound A5, melting point 135–136°, as impure dehydroisoandrosterone. Compound A26, melting point 174–175°, C₂₁H₃₂O₃, has been shown to be 11-ketopregnanolone by the identity of its infra-red spectrum with that of an authentic sample prepared by Dr. R. B. Turner. Confirmation of this identification has been obtained by chemical comparison of the compounds and of their derivatives.

Infra-red spectrometry can be used in the isolation of unknown compounds. It is frequently possible to recognize that a spectrum represents a mixture of a known product with an as yet unrecognized constituent. Further purification often leads to separation of the mixture and this process is facilitated by careful study of the infra-red spectrum. After successive fractionation and spectroscopic comparison have revealed no further change in spectrum, it is possible to identify the substance by comparison of the spectrum with that of a known pure compound. When the spectrum is not identical with that of any available product, considerable information about the substance still can be derived from a study of the spectrum. It is possible to determine whether the compound contains a hydroxyl group and to detect and locate the position in the molecule of the ethylenic double bonds and carbonyl groups (5–8). Such measurements can be made on a few micrograms of material (9) without loss of the sample.

DISCUSSION OF COMPOUNDS

Δ^1 -Androstenedione-3,17—The isolation of Δ^1 -androstenedione-3,17 (Compound A13a) and its isomer, Δ^4 -androstenedione-3,17 (Compound A14), raises the possibility that these compounds are interconvertible during the acid hydrolysis of the urine. When Δ^4 -androstenedione-3,17

was refluxed with sulfuric acid, only the Δ^4 isomer was recovered and there was no evidence for the formation of the Δ^1 compound. Therefore the provisional conclusion has been made that the Δ^1 isomer is a true hormonal metabolite and the isolation of other Δ^1 steroids can be anticipated. Although there is as yet no direct evidence, the Δ^1 and Δ^4 steroids may be intermediate in the transition of an aliphatic Ring A to the aromatic type, possibly through the $\Delta^{1,4}$ -dien-3-ones which have not as yet been isolated.

Testosterone—Testosterone has been isolated from human urine for the first time. It is remarkable that only trace amounts are excreted despite the administration of 90 mg. of the hormone per day for 45 days. About 50 per cent of the injected material was recovered from the urine in the form of recognizable steroid metabolites (10), but at most 10 to 20 mg. of unchanged testosterone were isolated, indicating that the metabolism of testosterone is accomplished rapidly and completely in the normal male.

Etiocholanol-3 β -one-17—With the isolation of etiocholanol-3 β -one-17 (Compound A19a), all four C₃ and C₅ isomers of the 3-hydroxy-17-keto metabolites of testosterone have now been realized. The presence of the other three C-19 isomers, androsterone (Compound A20), etiocholanolone (Compound A24), and isoandrosterone (Compound B3), has long been known and the failure to isolate the fourth isomer, etiocholanol-3 β -one-17 (Compound A19a), undoubtedly was due to the fact that it is excreted in trace amounts. The C-19 17-ketosteroids and the C-21 3,20-dihydroxy steroids now present an exact parallel. Since all four possible isomers at C₃ and C₅ are obtained from both series, it can be assumed that the normal metabolic fate of the precursors of these substances is the production of relatively large amounts of the 3 α -hydroxy compounds and much smaller amounts of their 3 β isomers.

11-Ketosteroids—11-Ketoetiocholanolone (Compound A28) was the first steroid isolated from urine with a carbonyl group at C₁₁ (11). Four additional substances with this characteristic feature of certain adrenocortical hormones are reported in this paper. Two of these metabolites, 11-ketopregnanolone (Compound A26) (12) and 11-ketoandrosterone (Compound A28a) (12), react with Girard's reagent, whereas the other two, 11-ketopregnanediol-3 α ,20 α (13) and 11-ketoetiocholanediol-3 α ,17 β , do not, and therefore are found in the non-ketonic fractions of urine. Together with pregnanetriol-3 α ,17,20 (14) these latter two compounds are the only urinary non-ketonic steroids that can with certainty be considered to be adrenal hormone metabolites.

11-Hydroxy Steroids—We consider that Δ^9 steroids, Δ^9 -androstenol-3 α -one-17 (15) and Δ^9 -etiocholenol-3 α -one-17 (15, 16), are transformation products formed by dehydration of 11 β -hydroxy steroids (17), and the

isolation of a Δ^9 compound is considered *prima facie* evidence for the presence of an 11β -hydroxy analogue. Confirmation of this assumption was achieved with the isolation of 11β -hydroxyandrosterone (1, 12, 18) and additional evidence has been obtained by the isolation of 11β -hydroxy-etiocholanolone. We sought this compound in the appropriate eluates from the chromatogram and, since we knew the infra-red spectrum, it was possible to recognize and identify the substance. The isolation and characterization by chemical means are described in this paper. The occurrence of 11β -hydroxyetiocholanolone in the urine of diseased individuals is of considerable metabolic and clinical significance and has been discussed elsewhere (10, 19, 20).

6-Hydroxy Steroids—The isolation of the two pregnanediolones, allo-pregnanediol- $3\alpha,6\alpha$ -one-20 (Compound A31) and pregnanediol- $3\alpha,6\alpha$ -one-20 (Compound A32), from pregnancy urine is of interest because they are the only urinary steroids with an oxygen function on C_6 . It is likely that they are true metabolic products; their precursors and the route of their metabolism remain to be established.

EXPERIMENTAL¹

Compound A13a, Δ^1 -Androstenedione-3,17—This compound was obtained from a pool of "early" fractions (2) obtained from the urines of a variety of healthy and diseased individuals. The material eluted with benzene from an aluminum oxide chromatogram exhibited infra-red spectra identical with that obtained from Δ^1 -androstenedione-3,17, generously given us by Dr. Carl Djerassi. The Δ^1 isomer was eluted just prior to its Δ^4 isomer, Δ^4 -androstenedione-3,17. In order to obtain a pure sample of Δ^1 -androstenedione-3,17, many recrystallizations from acetone-ligroin (b.p. 90°) and ether-ligroin were necessary to bring its melting point up to 138 – 142° ; $[\alpha]_D^{29} = +122^\circ \pm 7^\circ$ (2.93 mg. in 2.00 ml. of chloroform). Butenandt *et al.* (21) reported the melting point of 138 – 139° ; $[\alpha]_D^{23} = +119^\circ$ (alcohol); the sample supplied by Dr. Djerassi after sublimation and recrystallization from ether-ligroin melted at 143 – 146° . When this sample was mixed with the urinary compound, the mixture melted at 138 – 145° . The synthetic sample exhibited an absorption maximum at $231\text{ m}\mu$; $\log \epsilon = 4.08$; the urinary compound possessed a maximum at $232\text{ m}\mu$, $\log \epsilon = 4.10$. Δ^1 -3-Keto compounds characteristically exhibit an absorption maximum at a shorter wave-length than the Δ^4 -3-keto compounds (22). The infra-red spectra of both samples were identical in all respects.

¹ The microanalyses reported herein were performed by Dr. A. Elek, of The Rockefeller Institute for Medical Research, New York. The melting points were determined in a Hershberg melting point apparatus or on a Kofler micromelting point block and are correct to about $\pm 1^\circ$.

25 ml. of 10 per cent sulfuric acid solution were added to a boiling solution of 500 mg. of Δ^4 -androstenedione-3,17 in 15 ml. of ethanol. After heating $1\frac{1}{2}$ hours under a reflux, the solution was concentrated to dryness *in vacuo*. The residue was taken up in ether, the ether solution washed with sodium carbonate, and then with water until neutral. After evaporation of the ether, the residue, 486 mg., was chromatographed on 50 gm. of alumina. The chromatogram was developed with six 300 ml. portions of benzene-ligroin (1:1) and with fifteen 300 ml. portions of benzene. The first crystalline material obtained from the fourth benzene eluate was analyzed by infra-red spectroscopy and was found to be unchanged Δ^4 -androstenedione. Since Δ^1 -androstenedione-3,17 is eluted prior to its Δ^4 isomer, its presence was to be expected in the eluates preceding this fraction. However, these earlier eluates showed no specific infra-red absorption characteristic of either Δ^1 -androstenedione or Δ^4 -androstenedione. The crystalline material found in all of the subsequent benzene eluates was Δ^4 -androstenedione.

Compound A19a, Etiocholanol-3 β -one-17—This compound, isolated for the first time from human urine, was discovered by means of its infra-red spectrum in eluates from the α -ketonic fraction which precede androsterone. It was found in the urine of several normal men and women, pregnant women, and a variety of diseased individuals. The chromatographic eluates whose infra-red spectra indicated the presence of etiocholanol-3 β -one-17 were crystallized from ether-ligroin (b.p. 60°). Several crystalline fractions of wide melting point range (137–152°) were obtained and these were combined and rechromatographed on alumina. The crystalline eluates obtained from benzene-ligroin (1:1) and benzene, weighing 12 mg., were combined and recrystallized three times from ether-ligroin (b.p. 60°) to give 1.2 mg. of etiocholanol-3 β -one-17, melting at 154–155° with some softening between 148–150° (Kofler block). When mixed with an authentic sample (m.p. 150–152°) obtained through the kindness of Professor T. Reichstein, there was no depression in melting point.

This sample of etiocholanol-3 β -one-17 was combined with some lower melting samples and acetylated with acetic anhydride and pyridine at room temperature. The crystalline product was recrystallized three times from ligroin (b.p. 30°) and melted at 151–155° after preliminary softening at 148°. When mixed with an authentic sample of etiocholanol-3 β -one-17 acetate (m.p. 158–160°), the mixture melted at 155–158° (softening at 150°). The infra-red spectra of both samples were identical.

Although these samples of etiocholanol-3 β -one-17 were obtained from α -ketonic fractions, the compound forms an insoluble digitonide. 22 mg. of synthetic etiocholanol-3 β -one-17 were dissolved in 0.5 ml. of ethanol and treated with 122 mg. of digitonin dissolved in 1.3 ml. of ethanol and

0.2 ml. of water. Within a few minutes the digitonide precipitated. The next day it was filtered and washed several times with 90 per cent ethanol. The filtrate was diluted with water and extracted twice with ether. The ether-soluble residue, which corresponds to the α fraction, was an oil which weighed only 1 mg.

The digitonide weighed 99 mg. and was decomposed by boiling 1 hour in 0.5 ml. of pyridine. Ether extraction of the pyridine solution gave a crystalline product which weighed 18 mg. and did not depress the melting point of authentic etiocholanol-3 β -one-17.

*Compound A24a, Testosterone*²—This substance was detected by means of its infra-red spectrum in several eluates obtained during the chromatographic analysis of the α -ketonic fraction of the urine from two patients who had been injected with testosterone. From the small amount of material available it was possible to obtain only a few crystals from ether-ligroin (b.p. 30°) which melted at 143–151°, somewhat lower than testosterone, m.p. 154–155°. The infra-red spectrum provided evidence that this was an impure sample of testosterone. The oily mother liquor from these crystals showed a strong absorption in the ultraviolet at 240 m μ . Further chromatographic analysis and high vacuum sublimation of the material present in this mother liquor did not yield any additional crystalline testosterone, although its presence was indicated by its ultraviolet absorption at 240 m μ and its characteristic infra-red spectrum between 1185 and 875 cm.⁻¹.

Another attempt to isolate the testosterone was made by partitioning the oily mother liquors between benzene and concentrated hydrochloric acid. This procedure was successfully employed by David *et al.* (24) to isolate testosterone from horse testes and was also used by Prelog *et al.* (25) for the isolation of the androgenic substance from swine testes. 12 mg. of an oily fraction, whose spectroscopic characteristics indicated the presence of the hormone, were partitioned between benzene and concentrated hydrochloric acid. The oil was dissolved in 1 ml. of benzene and extracted five times with 0.5 ml. portions of concentrated hydrochloric acid. The residue remaining in the benzene showed a weak absorption at 229 m μ and its infra-red spectrum revealed no absorption characteristic of testosterone. The acid washes were diluted with water and then extracted with ether. The ether-soluble residue which exhibited a strong absorption at 242 m μ weighed 7 mg. and was chromatographed on alumina. The oil eluted with ether-benzene (1:3) and ether-benzene (1:1) weighed 3.14 mg. and showed an absorption maximum at 239 m μ , ϵ = 11,100 (ϵ_{238} for testosterone = 12,600). Although the infra-red spectrum

² The C₁₇-OH group of this compound is considered to be β -oriented; for a discussion of this configuration see (23).

again demonstrated the presence of testosterone, the oil could not be crystallized. The material (3 mg.) was then benzoylated with benzoyl chloride and pyridine and the oily product chromatographed on alumina. The oil (1 mg.) eluted with benzene could not be crystallized, but its infra-red spectrum indicated the presence of testosterone benzoate. It also absorbed strongly in the ultraviolet at 230 $m\mu$. The spectroscopic and chemical evidence accumulated established the presence of testosterone, although it was not obtained in a pure state.

Compound A26, Pregnanol-3 α -dione-11,20 (11-Ketopregnanol-3 α -one-20)—The isolation of Compound A26, $C_{21}H_{32}O_3$, m.p. 174–175°, from the urine of a girl with an adrenogenital syndrome (Case AHF13) has been reported (1), but because of the lack of material it was not possible at that time to identify the compound. When a synthetic sample of 11-ketopregnanolone was kindly made available to us by Dr. R. B. Turner, its infra-red spectrum was found to be identical to that of Compound A26.

Additional quantities of this substance were detected by infra-red spectrometry in some of the non-crystalline fractions eluted with benzene-ether (1:1) from this same patient. After several recrystallizations from ether and acetone-ether, the compound melted at 174.5–175°; $[\alpha]_D^{22} = +111^\circ \pm 3^\circ$ (3.06 mg. in 2.00 ml. of ethanol). The melting point was not depressed when mixed with an authentic synthetic sample. The rotation of Dr. Turner's sample was $[\alpha]_D^{22} = +112^\circ \pm 2^\circ$ (ethanol).

10 mg. of the urinary compound were oxidized at room temperature with chromic acid in glacial acetic acid solution. The triketone was recrystallized from ether-ligroin (b.p. 30°) and melted at 158–160.5°; $[\alpha]_D^{22} = +125^\circ \pm 4.5^\circ$ (2.23 mg. in 2.00 ml. of acetone). The reported (26) melting point is 154–156°; $[\alpha]_D^{20} = +119.5^\circ \pm 2^\circ$. There was no depression in melting point when mixed with an authentic sample of pregnanetrione-3,11,20 kindly made available to us by Dr. T. F. Gallagher. The infra-red spectra of both samples of pregnanetrione were identical.

Compound A27a; Etiocholanediol-3 α ,11 β -one-17 (11 β -Hydroxyetiocholanol-3 α -one-17)—The isolation of this compound from human urine was reported by us at the Laurentian Hormone Conference, September, 1947 (12, 13). Its presence in small amounts in urinary fractions was detected by means of its infra-red spectrum, which was similar to that of the synthetic sample of 11 β -hydroxyetiocholanolone kindly made available to us by Dr. L. H. Sarett (27). The compound was found in the urine of four patients with Cushing's syndrome (Cases CSF1, CSF2, CSF5, and CSF6), two patients with cancer (Cases CaP4 and CaBF16), and one patient with hypertension (Case HF4), and has not been detected in the urine of normal men and women. The small amounts which were obtained from individuals were pooled and rechromatographed on magnesium silicate-

Celite. 11-Hydroxyetiocholanolone was eluted from this adsorbent with benzene solution containing 30 per cent ether, together with its isomer, 11-hydroxyandrosterone. The mixture of these two C_5 isomers was crystalline and could be separated by fractional crystallization. Upon crystallization from acetone-ligroin, 11-hydroxyandrosterone crystallized out first. The residue in the mother liquor was recrystallized twice from acetone-ligroin (b.p. 30°) and once from acetone to give about 1 mg. of 11 β -hydroxyetiocholanol-3 α -one-17, m.p. $236-237^\circ$. The infra-red spectrum of this urinary compound was identical with that of the synthetic product and there was no depression in melting point when the two were mixed.

Compound A28a, Androstanol-3 α -dione-11,17 (11-Ketoandrosterone)—Since 11-ketoetiocholanolone (Compound A28) is excreted regularly in the urine of normal and diseased individuals in amounts of approximately 1 mg. per day, it was considered very likely that its diastereoisomer, 11-ketoandrosterone, was also a urinary metabolite of some of the adrenal hormones. In order to test this assumption, this hitherto unknown compound was prepared. 11-Hydroxyandrosterone acetate³ was oxidized with chromic acid in glacial acetic acid solution at room temperature. The resulting diketo acetate was purified by chromatography on alumina and the fraction eluted with benzene-ligroin (1:1) was recrystallized from ether. On the Kofler block the crystals changed form at $176-177^\circ$ and melted at $183.5-184.5^\circ$; $[\alpha]_D^{24} = +113^\circ \pm 4^\circ$ (4.95 mg. in 2.00 ml. of ethanol).

Analysis— $C_{21}H_{30}O_4$. Calculated, C 72.81, H 8.73; found, C 73.35, H, 9.06

14 mg. of the acetate were dissolved in 1 ml. of methanol and to the solution was added 0.1 ml. of water containing 10 mg. of potassium carbonate. After standing overnight, the solution was heated for half an hour on the steam bath. The solution was then poured onto ice and water and extracted three times with ether. The neutral ether-soluble residue was chromatographed on magnesium silicate-Celite (2). Some unhydrolyzed 11-ketoandrosterone acetate was found in those fractions eluted with 10 per cent-30 per cent ether in benzene, and 4 mg. of 11-ketoandrosterone were obtained in the fraction eluted with ether-benzene (1:1) and ether. The product was recrystallized from acetone-ligroin (b.p. 30°) and melted at $153.5-155^\circ$; $[\alpha]_D^{25} = +127^\circ \pm 11^\circ$ (1.73 mg. dissolved in 2.00 ml. of ethanol).

With the infra-red spectra of these substances on hand a search was now made for the presence of 11-ketoandrosterone in urinary fractions. It was detected in the eluates from the urines of many normal and diseased

³ We are especially grateful to Dr. R. L. Dorfman and to Dr. H. L. Mason for supplying us with considerable amounts of this rare compound.

persons, but its isolation in crystalline form proved to be very difficult. It was present in small amounts and was always accompanied by much larger amounts of its isomer, 11-ketoetiocholanolone (Compound A28). Fractions containing 11-ketoandrosterone were combined, submitted to another Girard separation, and rechromatographed several times on alumina and on magnesium silicate-Celite (from which it was eluted with 30 per cent-50 per cent ether solution in benzene). Although the presence of this compound was detected in many eluates by infra-red spectroscopy, they could not be crystallized. Therefore 22 mg. of the best material were acetylated with acetic anhydride in pyridine solution at room temperature and the product purified by chromatography on alumina. The oil eluted with benzene-ligroin (1:1) was crystallized from ligroin (b.p. 30°) as needles (2 mg.), m.p. 174–179° (Kofler block). When mixed with the synthetic sample, there was no depression in melting point. The infra-red spectra of the two samples were identical.

Compound A31, Allopregnanediol-3 α ,6 α -one-20, and Compound A32, Pregnanediol-3 α ,6 α -one-20—The isolation and characterization of Compound A31 possessing the empirical formula, $C_{21}H_{34}O_3$, have been described (1). This substance was shown to be allopregnanediol-3 α ,6-one-20 because it formed a diacetate, a monoxime, and was oxidized to allopregnanetrione-3,6,20. The compound was considered to have a 3 α -hydroxy group, since it was not precipitated by digitonin, but no configuration for the 6-hydroxyl was assigned. Dr. D. H. R. Barton of the Imperial College of Science and Technology, London, suggested to us that the method of molecular rotation differences (28) can furnish an answer to the question of the steric configuration at C₆ in Compound A31. He pointed out⁴ that the difference (+42°) between the molecular rotations of allopregnanediol-3 α ,6-one-20 (Compound A31) (+351°) and allopregnanol-3 α -one-20 (Compound A17) (+309°) is in the same direction and approximately of the same magnitude as the difference (+65°) between the molecular rotations of cholestanediol-3 β ,6 α (+154°) and cholestanol-3 β (+89°). The difference in molecular rotation between cholestanediol-3 β ,6 β (+57°) and cholestanol-3 β (+89°) is -32°. The same conclusions can be reached by considering the molecular rotation differences of the corresponding acetates. From this evidence, therefore, the hydroxyl group at C₆ of Compound A31 is in the α configuration.

We are also greatly indebted to Dr. W. Klyne, British Postgraduate Medical School, London, for having called our attention to the similarity between our Compound A32, $C_{21}H_{34}O_3$, and pregnanediol-3 α ,6 α -one-20 prepared by Moffett, Stafford, Linsk, and Hoehn (29). The melting points and rotations of both compounds are similar and the melting points

⁴ Private communication.

of their diacetates also agree. Furthermore, both compounds form a lower melting crystal modification when recrystallized from benzene. We secured through the kindness of Dr. W. M. Hoehn samples of pregnanediol- $3\alpha,6\alpha$ -one-20 and its diacetate, and, when these synthetic substances were compared directly by mixed melting point and by infra-red spectroscopy with Compound A32 and its diacetate, their identities were confirmed. Consequently the structure of Compound A32 has been definitely established.

α -Steroid Alcohols

Pregnanediol- $3\alpha,20\alpha$ -one-11 (*11-Ketopregnanediol- $3\alpha,20\alpha$*)—This compound is present in the urine of normal persons as well as in a variety of diseased individuals. Its isolation was reported simultaneously by Mason and by us at the Laurentian Hormone Conference, September, 1947 (13). Mason (30) isolated this compound as a metabolite of 11-dehydrocorticosterone which had been administered to a patient with Addison's disease. In the instance cited here, it was isolated from the urine of a woman with an adrenal hyperplasia (Case AHF1).

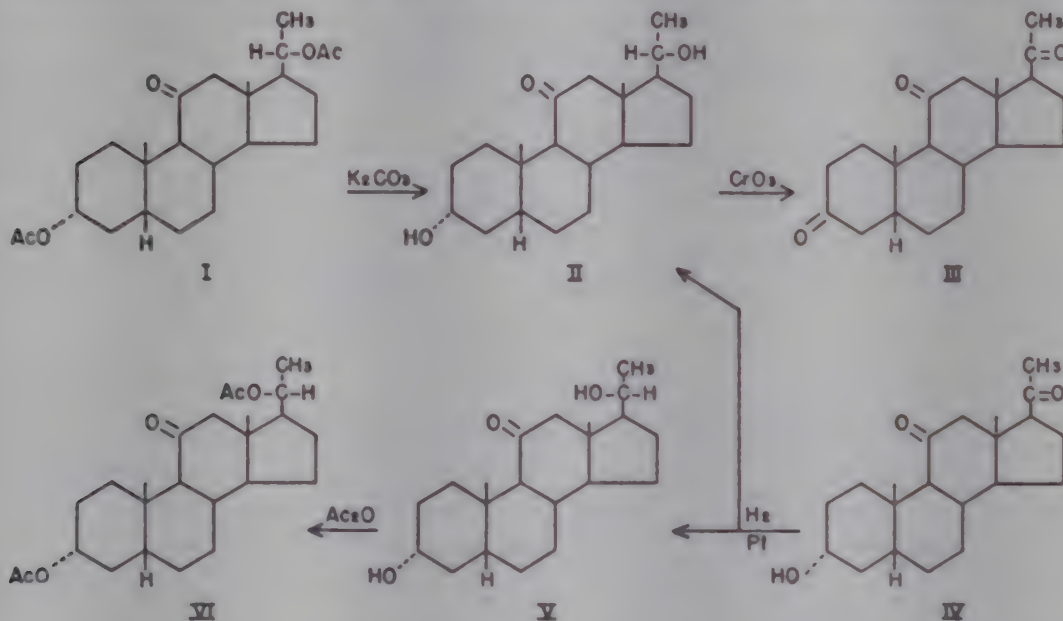
11-Ketopregnanediol does not react with Girard's reagent, and consequently it was found in the non-ketonic fraction. The non-ketonic fraction was partitioned into its alcoholic and non-alcoholic components by means of phthalic anhydride (2) and the alcoholic fraction so obtained was separated into α and β components with digitonin. The α alcohols were acetylated and submitted to systematic chromatographic analysis. Each eluate from these chromatograms was examined by infra-red spectroscopy in a search for components with characteristic carbonyl absorption (5), since C_{11} -ketones were expected in this non-ketonic fraction. In those eluates obtained from alumina with benzene-ligroin (1:1) a material was found with an absorption maximum at 1710 cm.^{-1} , indicative of a carbonyl group at C_{11} .

This substance (I) was crystallized from methanol and, after three additional recrystallizations, melted at $231\text{--}233^\circ$ (Kofler block); $[\alpha]_D^{24} = +56.7^\circ \pm 5^\circ$ (4.23 mg. in 2.00 ml. of ethanol). A small amount of (I) was analyzed for carbon-hydrogen but the result was inconclusive.

$C_{25}H_{38}O_5$. Calculated, C 71.73, H 9.15; found, C 70.36, H 8.86

The compound did not form a semicarbazone, additional evidence which pointed to a hindered 11-ketone. 16 mg. were dissolved in 2 ml. of hot methanol and treated with 0.2 ml. of water containing 20 mg. of potassium carbonate. After standing overnight, the methanol was removed *in vacuo* and the residue extracted into ether. The hydrolyzed product (II), after several recrystallizations from acetone, weighed 9 mg. and melted at 214--

217° (Kofler block). 5 mg. of this hydroxy ketone were oxidized with 0.15 ml. of 2 per cent chromic acid in glacial acetic acid solution at room temperature for 4 hours. The oxidation product (III) was recrystallized from ether-ligroin (b.p. 30°) and melted at 158–160°. It was identified as pregnanetrione-3,11,20 by mixed melting point with an authentic sample and by comparison of the infra-red spectra. The formation of this triketone together with the other evidence presented above indicated that the urinary compound (II) was 11-ketopregnanediol-3 α ,20. An attempt was made therefore to synthesize this unknown compound by partial hydrogenation of 11-ketopregnanolone (Compound A26) (IV). 35 mg. of 11-ketopregnanolone (m.p. 173–174°) dissolved in 1.5 ml. of acetic acid were



reduced with hydrogen in the presence of 8 mg. of prerduced Adams' catalyst. 2.8 ml. of hydrogen were absorbed (calculated for 1 mole, 2.6 ml.). After removal of the catalyst and the solvent, the crystalline residue (V) melted at 220–225°. This product was acetylated with acetic anhydride in pyridine solution and the diacetate (VI) obtained melted after recrystallization from acetone-methanol at 162–163° (Kofler block); $[\alpha]_D^{28} = +69.1^\circ \pm 7^\circ$ (2.89 mg. in 2.00 ml. of ethanol). Infra-red analysis confirmed that this diacetate (VI) was not identical with (I), which had been obtained from urinary sources. The failure to relate the urinary compound (I) to the synthetic 11-ketopregnanediol diacetate (VI) may be explained by the observation of Marker *et al.* (31), who showed that the pregnanediol produced by the catalytic reduction of pregnanol-3 α -one-20 had a steric configuration at C₂₀ opposite to that of the naturally occurring urinary pregnanediol.

We later learned from Dr. L. H. Sarett that he had carried out this reduction with large amounts of 11-ketopregnanolone (IV) and succeeded in

isolating from this reduction the two C_{20} -isomeric 11-ketopregnanediols (II and V).⁵ These two isomers together with their diacetates were generously made available to us by him. Direct comparison by mixed melting point and infra-red analysis indicated that the diacetate of the isomer Dr. Sarett obtained in larger amounts was identical with our synthetic substance (VI); melting point of Sarett's compound 158–160° (Kofler block); $[\alpha]_D^{28} = +64.5^\circ \pm 7^\circ$ (2.79 mg. in 2.00 ml. of ethanol).⁶ The diacetate of the second C_{20} isomer obtained from the reduction in 1.5 per cent yield was found by mixed point and infra-red analysis to be identical with the diacetate (I) of the urinary compound; melting point of Sarett's compound 230–231° (Kofler block); $[\alpha]_D^{22} = +62.5^\circ \pm 8^\circ$ (2.56 mg. in 2.00 ml. of ethanol).⁶ The free diol, pregnanediol-3 α ,20 α -one-11 (II), isolated from urine, melted as mentioned above at 214–217°; the sample obtained from Dr. Sarett melted at 214–216° (Kofler block).⁶

By comparing these hydrogenation results with those of Marker *et al.* (31) it is very likely that the 11-ketopregnanediol isolated from urine has the same steric configuration at C_{20} as the urinary pregnanediol-3 α ,20 α and therefore it is tentatively designated pregnanediol-3 α ,20 α -one-11 (II).

Etiocholanediol-3 α ,17 β -one-11 (11-Ketoetiocholanediol-3 α ,17 β)—The presence of this compound was first detected from its infra-red spectrum in the α non-ketonic alcoholic fractions obtained from a pool of urine of normal men. This fraction was acetylated and chromatographed; the compound was eluted from alumina with benzene-ligroin (1:5). Its infra-red spectrum indicated the presence of a carbonyl group at C_{11} (6) and the absorption in the region of 1185 to 875 cm^{-1} was similar to that given by 11-ketoetiocholanediol-3 α ,17 β -diacetate, generously made available to us by Dr. L. H. Sarett (33). The compound was crystallized from ether-methanol as plates melting at 182–183.5° (Kofler block); $[\alpha]_D^{26} = +46.9^\circ \pm 3^\circ$ (7.24 mg. in 2.00 ml. of chloroform). Sarett reported the melting point as 180–181°; $[\alpha]_D^{25} = +51.5^\circ$ (acetone). There was no depression in melting point when the two samples were mixed and their infra-red spectra were identical.

3.7 mg. of the diacetate were hydrolyzed with 0.5 ml. of 5 per cent methanolic potassium hydroxide. After heating 1 hour, the solution was concentrated to a small volume *in vacuo* and was diluted with water. The silky needles which precipitated were collected and recrystallized once more from methanol-water (1:2); m.p. 262–262.5° (Kofler block). When mixed with a sample of etiocholanediol-3 α ,17 β -one-11 prepared in the same way from Dr. Sarett's sample of the diacetate, there was no depression in melting point.

⁵ This work has now been published (32).

⁶ Melting point and rotation determined at the Sloan-Kettering Institute.

SUMMARY

1. Seven steroids have been isolated from human urine for the first time. Two compounds, androstanol-3 α -dione-11,17 and pregnanediol-3 α ,20 α -one-11, are new steroids and five are known: Δ^1 -androstenedione-3,17, etiocholanol-3 β -one-17, testosterone, etiocholanediol-3 α ,11 β -one-17, and etiocholanediol-3 α ,17 β -one-11.

2. Four urinary steroids which were partly characterized previously have been identified: Compound A5, dehydroisoandrosterone; Compound A26, pregnanol-3 α -dione-11,20; Compound A31, allopregnanediol-3 α ,6 α -one-20; and Compound A32, pregnanediol-3 α ,6 α -one-20.

3. The sequence of elution of urinary steroids from chromatographic columns has been related to chemical structure, and rules governing the order of elution have been presented. The value of infra-red spectrometry in the study of steroid metabolism has been demonstrated by the isolation and chemical identification of several steroids whose presence was predicted from specific absorptions in this region of the spectrum.

4. The metabolic significance of the urinary steroids described in this paper has been discussed.

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BIBLIOGRAPHY

1. Lieberman, S., Dobriner, K., Hill, B. R., Fieser, L. F., and Rhoads, C. P., *J. Biol. Chem.*, **172**, 263 (1948).
2. Dobriner, K., Lieberman, S., and Rhoads, C. P., *J. Biol. Chem.*, **172**, 241 (1948).
3. Noller, C. R., *J. Am. Chem. Soc.*, **61**, 2717 (1939).
4. Dobriner, K., Lieberman, S., Rhoads, C. P., Jones, R. N., Williams, V. Z., and Barnes, R. B., *J. Biol. Chem.*, **172**, 297 (1948).
5. Jones, R. N., Williams, V. Z., Whalen, M. J., and Dobriner, K., *J. Am. Chem. Soc.*, **70**, 2024 (1948).
6. Jones, R. N., Humphries, P., and Dobriner, K., *J. Am. Chem. Soc.*, **71**, 241 (1949).
7. Jones, R. N., and Dobriner, K., in Harris, R. S., and Thimann, K. V., *Vitamins and hormones*, New York, **7**, 293 (1949).
8. Jones, R. N., Humphries, P., Packard, E., and Dobriner, K., *J. Am. Chem. Soc.*, in press.
9. Hardy, J. D., Wilson, H., and Dobriner, K., *Federation Proc.*, **8**, 204 (1949).
10. Dobriner, K., and Lieberman, S., in Symposium on steroids, Madison, in press.
11. Lieberman, S., and Dobriner, K., *J. Biol. Chem.*, **166**, 773 (1946).
12. Lieberman, S., Fukushima, D. K., and Dobriner, K., *Federation Proc.*, **7**, 168 (1948).

13. Lieberman, S., and Dobriner, K., in Pincus, G., and Thimann, K. V., Recent progress in hormone research, New York, **3**, 71 (1948).
14. Butler, G. C., and Marrian, G. F., *J. Biol. Chem.*, **124**, 237 (1938).
15. Lieberman, S., Dobriner, K., and Rhoads, C. P., *Federation Proc.*, **6**, 270 (1947).
16. Dobriner, K., Lieberman, S., Hariton, L., Sarett, L. H., and Rhoads, C. P., *J. Biol. Chem.*, **169**, 221 (1947).
17. Shoppee, C. W., *J. Chem. Soc.*, 1134 (1946).
18. Mason, H. L., and Kepler, E. J., *J. Biol. Chem.*, **161**, 235 (1945).
19. Dobriner, K., Acta, Union internationale contra le cancer, Louvain, **6**, 315 (1948).
20. Dobriner, K., Lieberman, S., Abels, J., Homburger, F., Reifenstein, E. C., Jr., and Rhoads, C. P., *Cancer Res.*, in press.
21. Butenandt, A., Mamoli, L., Dannenberg, H., March, L., and Paland, J., *Ber. chem. Ges.*, **72 B**, 1617 (1939).
22. Woodward, R. B., *J. Am. Chem. Soc.*, **63**, 1123 (1941).
23. Fieser, L. F., and Fieser, M., Natural products related to phenanthrene, New York, 3rd edition, 325 (1949). Gallagher, T. F., and Kritchevsky, T. H., *J. Am. Chem. Soc.*, in press.
24. David, K., Dingemanse, E., Freud, J., and Laquer, E., *Z. physiol. Chem.*, **233**, 281 (1935).
25. Prelog, V., Tagmann, E., Lieberman, S., and Ruzicka, L., *Helv. chim. acta*, **30**, 1080 (1947).
26. Hegner, P., and Reichstein, T., *Helv. chim. acta*, **26**, 721 (1943).
27. Sarett, L. H., *J. Biol. Chem.*, **173**, 185 (1948).
28. Barton, D. H. R., and Klyne, W., *Chem. and Ind.*, 755 (1948).
29. Moffett, R. B., Stafford, J. E., Linsk, J., and Hoehn, W. M., *J. Am. Chem. Soc.*, **68**, 1857 (1946). Moffett, R. B., and Hoehn, W. M., *J. Am. Chem. Soc.*, **69**, 1995 (1947).
30. Mason, H. L., *J. Biol. Chem.*, **172**, 783 (1948).
31. Marker, R. E., Kamm, O., Wittle, E. L., Oakwood, T. S., Lawson, E. J., and Laucius, J. F., *J. Am. Chem. Soc.*, **59**, 2291 (1937).
32. Sarett, L. H., *J. Am. Chem. Soc.*, **70**, 1690 (1948).
33. Sarett, L. H., *J. Am. Chem. Soc.*, **69**, 2899 (1947).

THE DISTRIBUTION OF THE CHROMOPROTEINS, HEMOGLOBIN, MYOGLOBIN, AND CYTOCHROME c , IN THE TISSUES OF DIFFERENT SPECIES, AND THE RELATIONSHIP OF THE TOTAL CONTENT OF EACH CHROMOPROTEIN TO BODY MASS*

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It is recognized that the over-all energy metabolism and, hence, the oxygen consumption bear a relationship of proportionality, not to body mass, but to a fractional exponent of this quantity. The skin surface area has a similar mathematical relation to the body mass. Thus, approximately equal values for caloric output (heat loss) or for oxygen intake per unit time are obtainable in species of different size, when these measurements are related either to a fractional exponent of their body masses (2, 3) or to their measured or calculated surface areas (4, 5). One avoids the direction of thought into unproductive channels by not pursuing the academic arguments (2, 3) as to which of these bases of reference (fractional exponent of body mass or surface area) is the more correct or valid in expressing the basal metabolism. On the whole, similar deductions can be arrived at from each empirical reference base, and each suffers from similar disadvantages and defects, which are largely owing to the fact that they appear to support a concept, very probably incorrect, that all kinds of protoplasm are the same. Nevertheless, valuable clinical inferences have been made in man by the use of the surface area base, and, without undue faith in their ultimate significance, both surface area and fractional exponent of the body mass are of service in the interpretation of the over-all metabolism of different species.

With reference to specific metabolites or some of the agencies of metabolism and their relationship to body mass, the present knowledge is fragmentary. Dreyer *et al.* (6, 7), who first introduced the fractional expo-

* Most of this work was carried out under contract between the Office of Naval Research and the University of Pennsylvania. Preliminary reports have been made at the meetings of the Federation of American Societies for Experimental Biology at Atlantic City, March 15-19, 1948 (1), and at the Sir Joseph Barcroft Memorial Conference on Haemoglobin, Cambridge, England, June, 1948 (1).

ment of the body mass, $W^{0.70}$ to $W^{0.72}$, as a point of reference, deduced that the blood volumes (and, hence, the total hemoglobin content) of different species were the same when expressed on this basis. This deduction, as will be seen, appears untenable. In an early study by the writer (8), it was found that the output of the normal urinary pigment, urochrome (whose chemistry remains unknown), in several different sized species was directly proportional to their surface areas. The minimal nitrogen excretion (formerly designated "endogenous nitrogen"), attained on a high calorie-no protein regimen, has been shown to be constant per kilocalorie of heat loss in different species, and, therefore, proportional to an exponential function of the body weight, as $W^{0.7}$ (9, 10). The neutral sulfur excretion (a probable component of which is urochrome) in the fasting state has a similar relationship (*cf.* (2)). More recently, in analyses limited to epithelial tissues, Rosenthal and Drabkin (11) reported the suggestive observation that the concentration of cytochrome *c* (as in kidney cortex) had an inverse relationship to the body size of the species.

In view of the recognized importance of hemoglobin, myoglobin, and cytochrome *c* (all conjugated complexes of the common iron protoporphyrin type III) in oxygen homeostasis, it has appeared desirable to secure more exact and complete information upon the total amounts of these chromoproteins and their distribution in various tissues of different mammalian species. Such quantitative data could be expected to disclose the possible existence and the character of any relationships between each pigment and such factors as body mass. Aside from this, such information is valuable in developing valid concepts of the magnitudes of the metabolic transactions involved (1, 12), as well as in shedding further light on the discrete metabolic functions of the individual chromoproteins. Complete data upon the total hemoglobin, myoglobin, and cytochrome *c*, and the distribution of the latter, in the body of the rat have already been reported from this laboratory (13). In the present paper, selected comparative data on different species, gathered over a number of years, will be presented, and their interpretation discussed.

Methods

Analytical Procedures—The concentration of hemoglobin was determined spectrophotometrically as cyanmethemoglobin, with our usual constant ϵ ($c = 1$ mm per liter, $d = 1$ cm.) $= 11.5$ at wave-length $540\text{ m}\mu$ (14).¹ Since hemoglobin is restricted to the erythrocytes, the total hemo-

¹ In the notation ϵ ($c = 1$ mm per liter, $d = 1$ cm.), $\epsilon = (1/(c \times d)) \times \log I_0/I$, where the concentration, c , is expressed in mm per liter, the depth, d , in cm., the intensity of incident radiation, I_0 , is 1.0, and the intensity of the transmitted flux, I , is expressed as a fraction of unity. The various spectrophotometric constants used

globin may be obtained from the concentration of this chromoprotein per unit volume of blood times the total blood volume. In the rat (13), dog, and man, fairly reliable values for the latter are calculated from the plasma volume (measured by the dye, T1824, dilution method (16)) and the fraction of plasma, determined by hematocrit. For the cow and horse the evaluations of blood volume are less reliable, but are regarded as sufficiently close for present purposes. The values for blood volume, which have been used, are furnished in Column 1, Table IV.

A quantitative volumetric application (13) of Morgan's modification of Theorell's method (17) was adapted for the extraction of myoglobin.² For quantitative estimation of this pigment, the isolation procedure needs to be carried only to the stage of separation of contaminating hemoglobin, with solution of the myoglobin in 3 M phosphate buffer of pH 6.6 (13). The chromoprotein was converted to cyanmetmyoglobin by addition of ferricyanide and cyanide, and its concentration determined spectrophotometrically, with the constant ϵ ($c = 1$ mM per liter, $d = 1$ cm.) = 11.3 at wave-length 540 m μ (13, 15). As there is no indication at present that organs such as liver, spleen, and kidney may contain tissue hemoglobin analogues, myoglobin was assumed to be limited to skeletal, cardiac, and smooth muscle. The chromoprotein content of the latter was not determined, since it was regarded as negligible in comparison with the total myoglobin of the skeletal muscle. The total content of myoglobin was obtained from its concentration per unit mass of muscle times the total muscle mass. The values used for the latter are approximations. They varied in the different species between 36 and 45 per cent of the body mass and are given in Column 1, Table IV.

Cytochrome *c* in individual tissues was determined by the direct microspectrophotometric capillary cuvette-diaphragm technique of Rosenthal

by us for the hemin pigments have now been established upon, and are referable to, an iron basis (15). The spectrophotometric determination of cyanmethemoglobin and cyanmetmyoglobin is the most direct and unequivocal determination of hemin iron. The molecular magnitudes of reference are the 1 iron atom equivalent weights: 16,700 for hemoglobin (0.335 per cent Fe), 16,400 for myoglobin (0.340 per cent Fe), and 13,000 for cytochrome *c* (0.43 per cent Fe).

² In the original step of the procedure, small pieces of muscle, freed of ligaments and obvious fat, are ground finely and extracted in one-half their weight of water in a Waring blender. Extraction is continued overnight at refrigerator temperature, and the crude aqueous extract recovered by pressure exerted on the material placed in a muslin sac. In the case of cardiac muscle it has been demonstrated that, although myoglobin readily passed into the water, cytochrome *c* was totally retained in the press cake, from which it could be isolated, but only by the use of special extracting agents, dilute trichloroacetic (18) or sulfuric acid (19). This simple observation suggests that myoglobin may be relatively "free," whereas cytochrome *c* may be "bound" in the cellular structure.

and Drabkin (19). The organ content of this chromoprotein was obtained from the concentration per gm. of wet weight of tissue times the weight of the organ in gm. The summation of the values in the separate tissues yields the total content of cytochrome *c* in the body. This has been done for the rat (13) and for man (Table II). In these two species, the body content of cytochrome *c* has been found to be predicted with sufficient reliability from the content of the pigment in the skeletal muscle mass divided by the factor 0.8, or from its content in the combined muscle, heart, liver, and kidney mass, divided by the factor 0.93. In the dog, each of these methods of calculating the content of cytochrome *c* has been shown to yield the same results. In the larger species, cow and horse, it was assumed that a similar relationship of the content of this pigment in skeletal muscle to that of the whole body was valid, and the factor 0.8 was employed to approximate the total cytochrome *c* from its quantity in the muscle mass. The method of Rosenthal and Drabkin (19) and the original Keilin and Hartree procedure (18) have been found unreliable for the determination of the cytochrome *c* concentration (presumably very small) in human blood. Adsorption on other proteins or blood constituents may be the source of the difficulty, at present unresolved, but the recovery of cytochrome *c* added to the blood was poor. The cytochrome *c* content of human blood (Table II) is, therefore, uncertain.

Specimens of human tissues were largely obtained as promptly as possible after death from causes believed not to affect adversely the composition of the tissues subjected to analysis. The tissues of the cow and horse were obtained at the sacrifice of these animals, necessitated by injury or intercurrent disease. The body surface areas of the individuals (Column 1, Table IV) were calculated with the following formulas: rat, Rubner (20); dog, Cowgill and Drabkin (5); man, DuBois and DuBois (4); cow, Brody and Elting (21); and horse, Seuffert and Hertel (22).

Results

Table I contains comparative values, which illustrate the close parallelism of data on the rate of oxygen consumption (which reflects oxidative activity) and on cytochrome *c* concentration in tissues of the rat. Although exact comparison is hampered by the fact that oxygen consumption is referred to the usual "terminal" dry weight (in the Warburg analytical procedure), whereas the concentration of cytochrome *c* is on the basis of the initial dry weight, nevertheless, the relationship of the two is striking. Each is low in tissues with low oxidative ability (red blood corpuscles and skin), each has intermediate values in tissues with moderate or fairly high metabolism (skeletal muscle, brain, and liver), and each has high values in tissues which maintain a continuous high level of work,

requiring continuous large energy expenditure (kidneys and heart). Hence, with reference to their oxidative ability, the protoplasts of these various tissues are remarkably different. The range between the most and least active is some 200- to 300-fold: Q_{O_2} (heart)/ Q_{O_2} (erythrocytes) = 200 (from 20/0.1), and cytochrome *c* (heart)/cytochrome *c* (erythrocytes) = 242 (from 1.940/0.008).

The relationship of oxygen consumption and cytochrome *c* concentration is, doubtless, the basis for our earlier finding (23) that the determination of cytochrome *c* can be used as an index of the activity of cytochrome oxidase in tissues. The proportionality of oxygen consump-

TABLE I

Proportionality of Cytochrome c Concentration and Oxygen Consumption in Rat Tissues

Tissue	Q_{O_2} *	Cytochrome <i>c</i>	
		Per mg. dry weight†	Per gm. wet weight
		γ	γ
Red blood corpuscles.....	0.1	0.008	2.6
Skin.....	1-2	0.051	8
Skeletal muscle.....	6	0.381	98
Brain cortex.....	10	0.375‡	82‡
Liver.....	10+	0.607‡	223‡
Kidney cortex.....	20	1.433§	352§
Heart.....	20+	1.940§	447§
Retina.....	30		

* Microliters of O_2 consumed per mg. of dry weight of tissue per hour; approximate magnitudes related to "terminal" dry weight, *i.e.* dry weight after the analytical run of the tissue slice in the Warburg apparatus.

† For direct comparison with Q_{O_2} values.

‡ From data of Rosenthal and Drabkin (11).

§ From data of Crandall and Drabkin (13).

tion and the concentration of cytochrome *c* could not be wholly anticipated. This relationship presents a somewhat unusual feature, since, although cytochrome *c* is a member of a biocatalytic system, in the strict sense, it is the substrate for the enzyme, cytochrome oxidase (1). However, the finding can be explained by the generally accepted view-point that the oxidase is present in excess in tissues, while the concentration of cytochrome *c* is the limiting factor in the reaction of the two. This is fundamental in the interpretation and appreciation of any changes which may be induced (24, 25) in the cytochrome *c* concentration and content of organs and of the body.

In Table II are recorded our mean values for the cytochrome *c* concen-

tration and content of different human organs, calculated for a reference body mass of 70 kilos. The summation of these values yields a value of only 0.8 gm. for the total body content of this chromoprotein. The analytical results upon individual tissues are, with some exceptions, in es-

TABLE II
Total Cytochrome c Content of Adult Human Male

The values are calculated for a reference body weight of 70 kilos from the means of analytical data upon the individual tissues of three to eight subjects.

Organ	Weight of organ*	Per cent of body weight	Cytochrome c	
			Per gm. tissue, wet weight	Total in organ
	gm.		γ	mg.
Skeletal muscles.....	29,400	42.0	22	646.8
Heart.....	330	0.47	136	44.9
Skin†.....	12,600	18.0	2.1	26.5
Liver.....	1,610	2.3	15	24.2
Brain.....	1,400	2.0	14	19.6
Abdominal organs‡.....	1,400	2.0	6.5§	9.1
Blood.....	5,910	8.45	0.9¶	5.3
Kidneys.....	330	0.47	12	4.0
Lungs.....	490	0.70	1.6§	0.8
Skeleton.....	10,500	15.0	**	
Rest.....	6,030††	8.61	**	
Total.....	70,000	100		781

* Except for the blood and kidneys, organ weights are based on values reported by Skelton (26), adjusted to a total body mass of 70 kilos.

† "Whole" skin, including subcutaneous tissue.

‡ Exclusive of liver and kidneys.

§ From data of Rosenthal and Drabkin (11).

|| Value based on plasma volume of 3150 ml., numerically equal to 4.5 per cent of the body weight, 0.56 for fraction of plasma (from hematocrit), and a specific gravity for whole blood of 1.055. Skelton's value (26) for blood mass is 40 per cent too low.

¶ This value is questionable (see "Methods").

** Cytochrome c concentration not determined; assumed to be negligible in these tissues.

†† The difference between the total body mass of 70,000 gm. and the summation of the other components.

stantial agreement with corresponding data reported by Greenstein *et al.* (27). Minor discrepancies include the heart, in which our results are significantly higher, and the kidneys and lungs, in which they are somewhat lower. The concentration of this pigment in all human tissues is

appreciably lower than that in corresponding organs of the rat, reported by Crandall and Drabkin (13). However, a comparison of the data on the rat (last column, Table I) and man (fourth column, Table II) revealed that the differences in concentration of cytochrome *c* were very much greater in the case of some tissues than of others. Thus, rat liver and kidney had respectively 15- and 30-fold greater concentrations of the chromoprotein than the corresponding organs in man. On the other hand, the concentrations of cytochrome *c* were only 3 and 5 times greater in rat heart and skeletal muscle than in the same human tissues. Of main present pertinence was the total body content of cytochrome *c*. A 70 kilo man contained 781 mg. of the pigment (Table II), as compared with 14.4 mg. found earlier (13) in a 0.25 kilo rat. There was an obvious disproportionality between the body mass and cytochrome *c* ratios: mass (man)/mass (rat) = $70/0.25 = 280$; cytochrome *c* (man)/cytochrome *c* (rat) = $781/14.4 = 54$.

In Tables III and IV are presented summaries of some of our data from which deductions, regarded as significant, have been drawn. The data collected in Table III afford a comparison of the concentrations of myoglobin and cytochrome *c* in cardiac and skeletal muscle. From the information supplied on the organ weights, the content of these chromoproteins may also be readily calculated. This comparison of myoglobin and cytochrome *c* is of interest from several standpoints. In muscle tissue the two pigments, one functioning in the transport (28), the other in the utilization phase of oxygen homeostasis, exist side by side, and, hence, a direct comparison is afforded of their relative intracellular concentrations in the same tissues. Furthermore, heart muscle is an organ working continuously, with a relatively high rate of metabolic activity, whereas skeletal muscle, quite quiescent in the basal state (see Table V), is capable of periodic bursts of activity, involving at times enormous energy expenditures. It has already been stated that the concentrations of cytochrome *c* are not as far apart in the heart tissue of different sized species as they are in some of the other tissues. It may now be seen (Table III) that the concentration of cytochrome *c* concerned with cellular oxidative activity (or capacity) is 3 to 6 times higher in the organ of continuous work, the heart, than in skeletal muscle. The consistency of the interspecies data gives greater weight to this finding and the generalizations which may be drawn therefrom. The high concentration of cytochrome *c* in heart muscle is consonant with this organ's high rate of oxygen consumption (Table I), and also with recent demonstrations, by the technique of coronary sinus catheterization (29, 30), of the unusually high degree of deoxygenation of blood supplying the heart. It may also be noted that the concentration of this chromoprotein in muscle

tissue, with the exception of the horse, decreases with increasing size of the species.

The concentration of myoglobin (Table III), on the other hand, appears to have no systematic relationship to body size. It is high in those species (dog, horse) which either run fast or work hard, and it is low in

TABLE III
Comparative Values of Myoglobin and Cytochrome c in Heart and Skeletal Muscles of Different Species

Skeletal muscle mass in dog, man, and horse estimated as 36, 42, and 45 per cent of their respective body weights.

Species	Heart			Skeletal muscle			Ratio, concentrations in heart and skeletal muscle	
	Mass	Myo-globin	Cyto-chrome c	Mass	Myo-globin	Cyto-chrome c	Myo-globin	Cyto-chrome c
	gm.	mg. per gm.*	mg. per gm.*	kg.	mg. per gm.*	mg. per gm.*		
Rat†	0.73	0.91	0.447	0.113	0.89	0.098	1.0	4.6
Dog	51	2.1	0.230	2.286	5.1‡	0.048‡	0.4	4.8
	80	1.7	0.281	3.557	2.1‡	0.056‡	0.8	5.0
	120	1.7	0.228	5.328	2.8	0.052	0.6	4.4
Man	290		0.108	26.04		0.018		6.0
	320		0.148	28.14		0.024		6.2
	320	1.4	0.128	28.56	1.4	0.020	1.0	6.4
	330	1.1	0.137	29.40	1.1	0.023	1.0	6.0
	330	1.1	0.127	29.40	1.0	0.018	1.1	7.1
	335	1.0	0.146	30.24	1.0	0.022	1.0	6.6
	370		0.158	31.92		0.028		5.6
	375	1.3	0.138	32.76	1.4	0.024	0.9	5.8
Horse	2665	4.0	0.148	200	6.2	0.046	0.6	3.2
	3000	4.7	0.212	205	6.6‡	0.095‡	0.7	2.2
	2805	5.1	0.152	207	8.2	0.042	0.6	3.6
	2810	4.7	0.182	216	7.8	0.057	0.6	3.2
	2857	4.5	0.171	225	7.0	0.071	0.6	2.4
	2920	4.4	0.187	225	8.3‡	0.059‡	0.5	3.2

* Wet weight of tissue.
† Mean values from data of Crandall and Drabkin (13), calculated for an adult rat of 250 gm.
‡ These data were used to derive values for total myoglobin and cytochrome c in the dog and horse respectively in Table IV.

rat and man. Also, in the hard workers the myoglobin concentration is considerably higher in skeletal than in cardiac muscle, whereas in rat and man the concentration of this pigment is of a similar order of magnitude in the two types of muscle. The data may lend support to the view that myoglobin functions mainly in "oxygen debt" (28). On the basis

of the relative concentrations of myoglobin, disclosed by the data on the dog and horse, it is possible that the heart has less capacity for handling a developing oxygen debt than does skeletal muscle.

Our values for the concentration of myoglobin in the muscle tissues of the dog are lower than those reported in early studies by Whipple (31). This may be owing to the fact that contaminating hemoglobin, always present in aqueous extracts of muscle, is separated completely from the myoglobin in the present analytical technique. However, our values for myoglobin in man are also appreciably lower than those recently reported for human muscle by Biörck (32), who used a presumably reliable analytical procedure. The data in Table III also supply information as to the reproducibility of concentration values for the chromoproteins in individuals within one species. The values for cytochrome *c*, as already demonstrated for the rat (11, 13, 33), proved to be relatively constant for a particular tissue, and characteristic for each species. The concentration of myoglobin (and, hence, the total content of this pigment), though fairly uniform in the heart, was quite variable in skeletal muscle from subject to subject (dog, horse, Tables III and IV). This variability in myoglobin may be related to the factor of exercise conditioning thought to affect the level of myoglobin (31, 32), and a separate investigation of this aspect of the problem is contemplated.

In Table IV the most salient features of the relationship of the chromoproteins to the body weight in five different species are presented. Attention is directed to the following.

1. The content of each chromoprotein, hemoglobin, myoglobin, and cytochrome *c* (Column 3, Table IV), increases with increased body size, and it is obvious that the oxygen transport chemicals, especially hemoglobin, are present in enormously larger amounts (in terms of gm.) than is the representative of the oxygen utilization system. By assigning a value of unity to the content of cytochrome *c*, the relative abundance of the three chromoproteins may be calculated from the data in Column 3, and expressed as ratios of hemoglobin-myoglobin-cytochrome *c*. These ratios are 222:7:1 (rat), 467:25:1 (dog), 649:85:1 (dog), 1169:44:1 (man), 1786:247:1 (cow), 350:112:1 (horse), and 218:55:1 (horse). It becomes evident that the relative quantities of the three chromoproteins are quite different in the different species. Two sets of values are supplied for the dog and horse to indicate the variability in total myoglobin which, nevertheless, is unusually high in the horse. In this species, the myoglobin content is greater than 30 per cent of the hemoglobin content (compare 1867 gm. and 5805 gm., Column 3), whereas, in rat and man, total myoglobin is only 3 to 4 per cent of the total hemoglobin (from values in Column 3).

2. The evaluation of the chromoprotein content per kilo of body mass

TABLE IV

Relationships of Total Chromoproteins to Body Mass and Surface Area

In Column 1, *W* = body mass, *S* = surface area, *V* = blood volume, and *M* = skeletal muscle mass.

Species (1)	Chromoprotein (2)	Chromoprotein*				
		Total (3)	Per kilo (4)	Per sq.m. (5)	Per $W^{0.70}$ (6)	Per $W^{0.75}$ (7)
		gm. (3)	gm. (4)	gm. (5)	gm. (6)	gm. (7)
Rat;† <i>W</i> 0.250 kilos, <i>S</i> 0.0361 sq.m., <i>V</i> 19.2 ml., <i>M</i> 0.113 kilos	Hemoglobin	3.19	12.76	88.3	8.42	9.01
	Myoglobin	0.101	0.404	2.8	0.267	0.286
	Cytochrome c‡	0.0144	0.056	0.399	0.038	0.041
Dog; <i>W</i> 9.88 kilos, <i>S</i> 0.523 sq.m., <i>V</i> 790 ml., <i>M</i> 3.56 kilos	Hemoglobin	138.3	14.00	264.4	27.8	24.8
	Myoglobin	7.5	0.76	14.3	1.51	1.34
	Cytochrome c§	0.249	0.025	0.476	0.050	0.045
Dog; <i>W</i> 6.35 kilos, <i>S</i> 0.330 sq.m., <i>V</i> 508 ml., <i>M</i> 2.29 kilos	Hemoglobin	88.9	14.00	269.4	24.2	22.2
	Myoglobin	11.7	1.84	35.5	3.18	2.93
	Cytochrome c§	0.137	0.022	0.415	0.037	0.034
Man; <i>W</i> 70 kilos, <i>S</i> 1.87 sq.m., <i>V</i> 5600 ml., <i>M</i> 29.40 kilos	Hemoglobin	912.8	13.04	488.1	46.8	37.7
	Myoglobin	34.7¶	0.50	18.6	1.77	1.44
	Cytochrome c‡	0.781	0.011	0.417	0.040	0.032
Heifer; <i>W</i> 182 kilos, <i>S</i> 2.92 sq.m., <i>V</i> 14,560 ml., <i>M</i> 81.9 kilos	Hemoglobin	2215.0	12.17	758.6	57.9	44.8
	Myoglobin	307.0	1.69	105.1	8.03	6.20
	Cytochrome c§	1.24	0.0068	0.424	0.032	0.025
Horse; <i>W</i> 500 kilos, <i>S</i> 6.62 sq.m., <i>V</i> 45,000 ml., <i>M</i> 225 kilos	Hemoglobin	5805.0	11.61	876.9	75.1	55.3
	Myoglobin	1867.5	3.74	282.1	24.16	17.79
	Cytochrome c§	16.6	0.033	2.51	0.215	0.158
Horse;** <i>W</i> 455 kilos, <i>S</i> 6.23 sq.m., <i>M</i> 205 kilos	Myoglobin	1345.2	2.96	215.9	18.50	13.67
	Cytochrome c§	24.3	0.053	3.90	0.334	0.247

* For concentration of chromoproteins see Tables I to III.

† From data of Crandall and Drabkin (13).

‡ Values based on complete analyses of individual organs.

§ Values based on (total muscle cytochrome c) 0.8. In the dog, similar values were derived from (total cytochrome c in muscles, heart, liver, and kidneys) 0.93.

|| Based on a concentration of 16.3 gm. per 100 ml. of blood (34).

¶ Owing to variations in the myoglobin content, a value up to 41 gm. could be calculated for the total myoglobin of an adult man of 70 kilos.

** Steeplechase thoroughbred, out of training.

(Column 4, Table IV) discloses that, in the case of hemoglobin, closely similar values (mean = 12.7 ± 0.7 gm. of hemoglobin per kilo of body mass) are obtained in the different species. Hence, *the content of hemoglobin is directly proportional to body mass.*

3. Columns 5, 6 and 7 of Table IV contain the values of chromoprotein content, calculated respectively per sq. m. of body surface area and per fractional exponent of the body mass, $W_{\text{kg.}}^{0.7}$ (2, 5) and $W_{\text{kg.}}^{0.75}$ (3). An examination of the values in these columns makes it clear that a similar, significant deduction may be made by the use of any one of the three bases of reference, namely that the cytochrome *c* content is, in four of the five species (rat, dog, man, and cow), quite constant either per sq.m of estimated body surface or per fractional exponent of the body weight. Therefore, *the total content of cytochrome c is proportional to an exponential function of the body mass, close to $W^{0.667}$, which is theoretical for the relationship of surface to volume.* However, an extension of this deduction as a generalization to all mammalian species is prohibited, since the horse is exceptional. The body of the horse appears to contain too much cytochrome *c* in comparison with those of the other species examined.

DISCUSSION

The relationships of hemoglobin and cytochrome *c* to body mass, though different, are equally remarkable. The finding that the total content of hemoglobin in different species is directly proportional to their body size seems to be consonant with the oxygen transport function of this chemical. The size of biological transport systems, functioning spatially, is apparently accommodated to the dimensions of the space they supply. Calculations, made by the writer (12) a number of years ago, from available information on the size of the mammalian erythrocyte, its water content, and the molecular volume of hemoglobin (based on dimensional data of Perutz (35)), indicated that the intracellular concentration of hemoglobin of the order of 34 gm. per 100 ml. is close to the maximum possible, under normal conditions. This must have consequences. An increase in hemoglobin above normal levels would demand either a change in the nature of the red blood cells or an increase in their number. Also, normally, owing to rough similarity in the size of the erythrocytes and in the relative volume of cells to plasma in different mammals, the relation of proportionality of hemoglobin content to body mass may be expected to apply to that of erythrocyte content and blood volume.

Though not applicable to all species, the proportionality of cytochrome *c* content to surface area or to a fractional exponent of the body mass, as $W_{\text{kg.}}^{0.7}$, is, nevertheless, one of the most striking relationships thus far uncovered for a cellular chemical. The involvement of cytochrome *c* in

the mechanics of cellular oxygen use lends especial cogency to the above relationships, since the basal energy metabolism as well as the basal rate of oxygen consumption is also proportional in different species to their surface areas or to $W_{kg.}^{0.7}$.

The chromoprotein-body mass relations, implied by the data in Table IV, may be expressed mathematically by so called heterogonic equations (equations expressing "degree of disproportionality" or of "similitude" (2, 3, 36)) of the general form $Y = aX^n$ or $a = Y/X^n$. In such an equation Y and X represent two separate quantities or rates, which may be disproportional to each other, but for which similitude can be obtained by means of the constants a and n . In the present work, Y is the quantity of chromoprotein in gm. and X^n is the body mass in kilos, raised to an appropriate power, n . In the case of hemoglobin (Column 4, Table IV) n is obviously close to unity and X^n may be substituted by $W_{kg.}^{1.0}$, while $a = 12.7$ gm., the mean of the values in Column 4. Thus, we may write

$$\frac{Hb}{W_{kg.}^{1.0}} = 12.7 \text{ gm.} \quad (1)$$

In the case of cytochrome c (Column 6, Table IV) $n = 0.7$ and X^n may be replaced by $W_{kg.}^{0.7}$, while $a = 0.039$ gm., the mean of the values in Column 6 for rat, dog, man, and cow. For the horse, $a = 0.275$ gm., an appreciably higher value. The relationships may be written

$$\frac{\text{Cytochrome } c \text{ (rat, dog, man, cow)}}{W_{kg.}^{0.7}} = 0.039 \text{ gm. or } 3.0 \mu M \quad (2)$$

$$\frac{\text{Cytochrome } c \text{ (horse)}}{W_{kg.}^{0.7}} = 0.275 \text{ gm. or } 21.2 \mu M \quad (3)$$

From pertinent data in the literature on the basal metabolic rates of the rat, dog, man, cow, and horse, the writer has obtained the following relation:

$$\frac{\text{Basal metabolic rate}}{W_{kg.}^{0.7}} = 86.5 \text{ kilocalories per day or } 0.001 \text{ kilocalorie per sec.} \quad (4)$$

The thermal equivalent of oxygen is 4.825 kilocalories per liter at the average basal R.Q. of 0.82. With this factor, equation (4) may be modified to

$$\frac{\text{Basal rate of oxygen consumption}}{W_{kg.}^{0.7}} = 0.207 \text{ ml. per sec. or } 9.2 \mu M \text{ per sec.} \quad (5)$$

Although it may not be strictly correct to talk about a "turnover rate" for cytochrome c , equations (2), (3), and (5) establish empirical relation-

ships between the rate of oxygen consumption and the quantity of this chromoprotein. In the rat, dog, man, and cow, the relationship is the same, namely 3 μM of cytochrome *c* to 9 μM of O_2 per second (basal state), whereas in the horse it is 21 μM of cytochrome *c* to 9 μM of O_2 per second (basal state). Adolph (36) has used the data on hemoglobin, myoglobin, and cytochrome *c* content in the writer's preliminary report (1), and has chosen to lump together the data on the cytochrome *c* of the horse with that of the other species by the device of appropriately raising the value of n in the heterogonic equation. This is mathematically permissible, and suggests that rough similitude is possible for all the species. However, at this stage, it appears more objective to accept and deal with, rather than to mask, the dissimilarity of the horse.

It may be pointed out that the similarity of energy metabolism and oxygen consumption per $W_{\text{kg}}^{0.7}$ in different species is regarded as applicable only to measurements in the basal state. However, it seems improbable that the relationship of cytochrome *c* content to the same base has similar connotations. Attention is again called to the fact that 80 per cent of the total cytochrome *c* is in skeletal muscle, the tissue of periodic, mechanical work. $W_{\text{kg}}^{0.7}$ is thought to represent that fraction of the body mass which is "metabolically effective" (2, 3). It becomes pertinent to inquire to what extent the skeletal muscles (which form 36 to 42 per cent of the body weight), and the cytochrome *c* contained therein, participate in the basal metabolism and oxygen consumption. To answer this question, the writer has assembled in Table V reliable recent data, obtained by means of vascular catheterization (30, 37-40), upon the basal metabolism of individual human organs. When the separate data on brain, heart, kidneys, and liver, which may be regarded as organs of continuous metabolic activity, are brought together and summated, an important deduction becomes inescapable. In the basal state, these organs, which comprise only some 5 per cent of the body mass, take some 70 per cent of the total blood flow and are responsible for 70 per cent of the metabolism (*i.e.* oxygen consumption). Clearly, shifts in the allocation of metabolic work (as well as in metabolic pattern) must occur in the periodic changes from a basal or resting to a working state, involving muscular activity. Skeletal muscle is known to be supplied with a unique capillary bed, which can open up to allow large increases in blood flow, and, hence, indirectly in oxygen consumption. In the basal state, the skeletal muscle is truly quite inactive metabolically, and this large fraction of the body mass is responsible for only 15 per cent or less of the total metabolic activity. However, the relative contribution of muscle tissue to metabolism becomes appreciably greater when mechanical work is done. This can only mean that "effective metabolic mass" is of very different magnitude

TABLE V

Allocation of Total Blood Flow (Cardiac Output) and Oxygen Consumption in Man in Basal State

Organs which form about 5 per cent of the total body mass are responsible for 70 per cent of the basal metabolism and take about 70 per cent of the cardiac output.

Tissue or organ*	Per cent of body mass of 70 kilos	Blood flow through organ	O ₂ consumption by organ	Visceral blood flow, per cent of cardiac output (total flow) of 5390 ml. per min.	Visceral O ₂ consumption, per cent of total O ₂ consumption of 248 ml. per min.
	<i>per cent</i>	<i>ml. per min.</i>	<i>ml. O₂ per min.</i>		
Whole body	100.00	5390†	248.0	100.00	100.00
Brain	2.00	756‡	47.6‡	14.03	19.2
Heart	0.47	215§	25.8§	3.99	10.4
Kidneys	0.47	1248	21.7	23.05	8.8
Liver	2.30	1683¶	79.7¶	31.21	32.1
Total, brain, heart, kidneys, liver	5.24	3902	174.8	72.3	70.5
Rest of body by difference	94.76	1488	73.2	27.7	29.5
Muscles	42.00	659** 824††	32.4** 40.6††	12.2 15.3	13.1 16.4

* For organ weights see Table II.

† Calculated from the basal oxygen consumption rate of 248 ml. per minute and an arterial venous difference in oxygen of 4.6 volumes per cent.

‡ Calculated from data of Kety and Schmidt (37).

§ Calculated from data of Bing *et al.* (30).

|| Calculated from averages of data of Chasis *et al.*, Bradley *et al.*, and Clark *et al.* (38).

¶ Calculated from averages of data of Myers and Bradley *et al.* (39). The values for oxygen consumption and blood flow are not attributable exclusively to liver, but include some other splanchnic tissues.

** Calculation based on assumption that the metabolism of resting muscle is the same as that of the "remaining organs" (*i.e.* organs exclusive of brain, heart, kidney, and liver).

†† Calculation based on assumption that the metabolism of resting muscle is 20 per cent higher than that of the "remaining organs." The value of 824 ml. per minute for blood flow through the skeletal muscle mass agrees well with that of 798 ml. per minute, based on the determination of Asmussen *et al.* (40) on the leg muscles of man.

in the basal and working states. Since 80 per cent of the cytochrome *c* is in skeletal muscle, one may conclude that only a fraction of the total cytochrome *c* activity is exhibited in the basal state, and that the total content of cytochrome *c* must be related to a factor such as *metabolic ca-*

capacity rather than *basal metabolism*. The metabolic capacity, evaluated from the rates of oxygen consumption during prolonged muscular work or at peak effort, possible only for brief intervals of time, is respectively 10- to 20-fold greater than the basal metabolism, *i.e.* an increase from 9 to 90 and 180 μM of O_2 per second. The finding of constancy of cytochrome *c* content per $W_{\text{kg.}}^{0.7}$ in rat, dog, man, and cow suggests, therefore, that in these species there is a similar relationship of oxygen consumption capacity to basal oxygen consumption, which may be designated as the *index of the expansibility of the metabolism*. In these species the degree of expansibility of metabolism with muscular work should be the same, namely 10/1 to 20/1 (from the above relative values of O_2 consumption). Present information (2) does not allow an unequivocal conclusion that the horse has a greater metabolic capacity than the other species. However, of the five species examined, the horse is the most representative as an organism which does long continued, hard muscular work. Consonant with this, is not only the high cytochrome *c* content but also the very high myoglobin content of horse skeletal muscle. While the data suggest that the "turnover rate" with respect to oxygen and cytochrome *c* is lower in the horse than in the other species, the interpretation could also be made that in the horse there is a greater potentiality for an expansion of cytochrome *c* function, in the sense that less strain is placed during prolonged muscular work on the mechanism of oxygen utilization.

To return to oxygen transport, a further deduction of interest may be drawn from the data in Table V. The proportionality of the oxygen consumption of tissues with the rate of the blood flow through them suggests that the *function of the carrier, hemoglobin, may be regarded as passive, the rate of oxygen supply being largely dependent on circulatory dynamics (cardiac rate, distribution of blood supply, etc.)*.

The data in Column 4, Table IV, upon myoglobin suggest a tendency for an actual increase in the content of this pigment per kilo of body weight with increasing size of species. Rough similitude for the myoglobin content in the different species can be shown from our data, as Adolph has pointed out (36), by the use of an exponent, n , greater than unity in the heterogonic equation. But, owing to the variability in the myoglobin content (dog and horse, Table IV), the establishment of a reliable value of n is not possible, and, in the case of this chromoprotein, stretching of the mathematical treatment to establish similarity would seem to be of questionable value.

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SUMMARY

1. The total body content of hemoglobin, myoglobin, and cytochrome *c* has been determined in five species, rat, dog, man, cow, and horse, and an interspecies comparison has been made. The comparative data have disclosed significant relationships of these chromoproteins to body mass.

2. In all the species, the quantity of hemoglobin, the oxygen transport chemical, has been demonstrated to be relatively constant per kilo, and, hence, directly proportional to body mass. In the different species, the relationship is $Hb/W_{kg.}^{1.0} = 12.7$ gm. The rate of oxygen supply appears to be largely dependent on circulatory factors, and the function of the oxygen carrier may be regarded as an essentially passive one.

3. The total content of cytochrome *c*, involved in cellular oxygen utilization, has been shown, on the other hand, to be proportional, in four of the five species, to the estimated body surface area or to $W_{kg.}^{0.7}$. The relationship, in rat, dog, man, and cow, is (cytochrome *c*)/ $W_{kg.}^{0.7} = 3.0$ μM . Correlated with the above, (basal energy metabolism)/ $W_{kg.}^{0.7} = 0.001$ kilocalorie per second or 9.2 μM of O_2 per second, while (metabolic capacity)/ $W_{kg.}^{0.7} = 0.01$ to 0.02 kilocalorie per second or 90 to 180 μM of O_2 per second. In the horse, the relationship of cytochrome *c* to body mass was found to be different from the other species, and was comparatively too high per $W_{kg.}^{0.7}$.

4. In all the species examined, the concentration of cytochrome *c* in cardiac muscle, a tissue of continuous work, was higher by a factor of 3 to 6 than in skeletal muscle, which is subject to periodic bursts of activity involving large energy expenditures. The myoglobin concentration, on the other hand, in two of the species (dog and horse), was higher in skeletal muscle than in heart.

5. The significance of the deductions drawn from the observations has been discussed. Attention has been directed to limitations in the commonly used basal metabolic state reference base, as well as in the concept of "metabolically effective body mass."

BIBLIOGRAPHY

1. Drabkin, D. L., *Federation Proc.*, **7**, 483 (1948). Drabkin, D. L., in Roughton, F. J. W., and Kendrew, J. C., *Haemoglobin*, London, 35 (1949).
2. Brody, S., *Bioenergetics of growth*, New York (1945).
3. Kleiber, M., *Physiol. Rev.*, **27**, 511 (1947).
4. DuBois, D., and DuBois, E. F., *Arch. Int. Med.*, **15**, 868 (1915); **17**, 863 (1916).
5. Cowgill, G. R., and Drabkin, D. L., *Am. J. Physiol.*, **81**, 36 (1927).
6. Dreyer, G., and Ray, W., *Proc. Roy. Soc. London, Series B*, **82**, 545 (1910); *Phil. Tr. Roy. Soc. London, Series B*, **201**, 133 (1910); **202**, 191 (1910).
7. Dreyer, G., Ray, W., and Walker, E. W. A., *Proc. Roy. Soc. London, Series B*, **86**, 39, 56 (1912-13).

8. Drabkin, D. L., *J. Biol. Chem.*, **75**, 443 (1927).
9. Terroine, E. F., and Sorg-Matter, H., *Compt. rend. Acad.*, **186**, 1017 (1928); *Arch. internat. physiol.*, **30**, 115, 126 (1928).
10. Smuts, D. B., *J. Nutr.*, **9**, 403 (1935).
11. Rosenthal, O., and Drabkin, D. L., *J. Biol. Chem.*, **150**, 131 (1943).
12. Drabkin, D. L., *Science*, **101**, 445 (1945).
13. Crandall, M. W., and Drabkin, D. L., *J. Biol. Chem.*, **166**, 653 (1946).
14. Drabkin, D. L., and Austin, J. H., *J. Biol. Chem.*, **112**, 51 (1935-36).
15. Drabkin, D. L., *Am. J. Med. Sc.*, **209**, 268 (1945); *Federation Proc.*, **4**, 88 (1945).
16. Gregersen, M. I., Gibson, J. J., and Stead, E. A., *Proc. Am. Physiol. Soc.*, *Am. J. Physiol.*, **113**, P54 (1935). Gibson, J. G., 2nd, and Evans, W. A., Jr., *J. Clin. Invest.*, **16**, 301 (1937).
17. Morgan, V. E., *J. Biol. Chem.*, **112**, 557 (1935-36). Theorell, H., *Biochem. Z.*, **252**, 1 (1932); **263**, 46 (1934).
18. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **122**, 298 (1937).
19. Rosenthal, O., and Drabkin, D. L., *J. Biol. Chem.*, **149**, 437 (1943).
20. Rubner, M., *Z. Biol.*, **19**, 535 (1883).
21. Brody, S., and Elting, E. C., *Missouri Agr. Exp. Sta., Res. Bull.* 89 (1926).
22. Seuffert, R. W., and Hertel, F., *Z. Biol.*, **82**, 7 (1924-25).
23. Rosenthal, O., and Drabkin, D. L., *Am. J. Med. Sc.*, **204**, 914 (1942); *Federation Proc.*, **2**, 69 (1943).
24. Drabkin, D. L., *J. Biol. Chem.*, **182**, 335 (1950).
25. Drabkin, D. L., *J. Biol. Chem.*, **182**, 351 (1950).
26. Skelton, H., *Arch. Int. Med.*, **40**, 140 (1927).
27. Greenstein, J. P., Werne, J., Eschenbrenner, A. B., and Leuthardt, F. M., *J. Nat. Cancer Inst.*, **5**, 55 (1944).
28. Millikan, G. A., *Physiol. Rev.*, **19**, 503 (1939).
29. Goodale, W. T., Lubin, M., Eckenhoff, J. E., Hafkenschiel, J. H., and Banfield, W. G., Jr., *Am. J. Physiol.*, **152**, 340 (1948).
30. Bing, R. J., Hammond, M. H., Handelsman, J. C., Powers, S. R., Spencer, F. C., Eckenhoff, J. E., Goodale, W. T., Hafkenschiel, J. H., and Kety, S. S., *Am. Heart J.*, **38**, 1 (1949).
31. Whipple, G. H., *Am. J. Physiol.*, **76**, 693 (1926).
32. Björck, G., *Acta Cardiol.*, **3**, 223 (1948); *Acta med. Scand.*, **133**, suppl. 226 (1949).
33. Drabkin, D. L., *J. Biol. Chem.*, **171**, 395, 409 (1947).
34. Drabkin, D. L., *Am. J. Med. Sc.*, **215**, 110 (1948).
35. Perutz, M. F., *Nature*, **143**, 73 (1939); **149**, 491, (1942); **150**, 324 (1942).
36. Adolph, E. F., *Science*, **109**, 579 (1949).
37. Kety, S. S., and Schmidt, C. F., *J. Clin. Invest.*, **27**, 476 (1948).
38. Chasis, H., Redish, J., Goldring, W., Ranges, H. A., and Smith, W. H., *J. Clin. Invest.*, **24**, 583 (1945). Bradley, S. E., and Halperin, M. E., *Federation Proc.*, **6**, 80 (1947). Clark, J. K., and Barker, H. G., *Federation Proc.*, **8**, 26 (1949).
39. Myers, J. D., *J. Clin. Invest.*, **26**, 1130 (1947). Bradley, S. E., Ingelfinger, F. J., Bradley, G. P., and Curry, J. J., *J. Clin. Invest.*, **24**, 890 (1945).
40. Asmussen, E., and Nielsen, M., *Acta physiol. Scand.*, **12**, 171 (1946).

CYTOCHROME *c* METABOLISM AND LIVER REGENERATION. INFLUENCE OF THYROID GLAND AND THYROXINE*

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In previous reports from this laboratory, results have been presented of studies of the effect of various factors, such as diet (3) and anoxia (4), on the metabolism of cytochrome *c*, investigated by means of partial hepatectomy or liver lobectomy, used as a quantitative metabolic technique (5). In the preceding communication (6), a survey of the cytochrome *c* concentration of various tissues and of the total body content of the major chromoproteins in different mammalian species has disclosed the following relationships: (1) The levels of concentration of cytochrome *c* in individual tissues are directly correlatable with the "respiratory" or oxidative activity. (2) There is (in four of the five species examined (6)) a striking proportionality of the body content of this chromoprotein to the surface area or to a fractional exponent of the body mass, $W_{\text{kg}}^{0.7}$, to which the basal rates of energy metabolism and of oxygen consumption are also proportional. Since hormones, especially thyroxine, have long been regarded as at least partially responsible for metabolic rate, the above findings influenced the direction of our studies of chromoproteins towards an investigation of a possible relation between hormonal factors and cytochrome *c* function or metabolism. In this paper, results bearing upon the influence of the thyroid gland and thyroxine on cytochrome *c* will be reported; in the following paper (7) data on the influence of the adrenals will be presented.

As far as the writer is aware no evidence has thus far been available from which valid inferences could be drawn as to the mechanism whereby a hormone like thyroxine regulates the rate of oxygen consumption of tissues and of bodies. With reference to a relationship of hormones to cytochrome *c*, pertinent information in the literature is scanty and inconclusive. Tipton and colleagues (8, 9) have presented some evidence for

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opposite effects of thyroid feeding and adrenalectomy on cytochrome oxidase activity. Tissières (10, 11) has studied the influence of the thyroid gland on cytochrome *c*, but his results were limited to a single tissue, skeletal muscle. Before the publication of our preliminary reports (1, 2) only the original communication by Tissières (10) was available to us. In this, the analytical values for the cytochrome *c* concentration of normal muscle appeared unacceptably low. In his later publication (11), Tissières, now using the Rosenthal and Drabkin analytical procedure for cytochrome *c* (12), obtained results in good agreement with ours for normal muscle tissue. Since cytochrome *c* is a constituent of all aerobic cells, to be of conclusive significance, a hormonal effect upon this chromoprotein must be demonstrated for all tissues. In our work, we have collected data on the cytochrome *c* concentration, before and after appropriate experimental procedures, in liver, kidneys, heart, and skeletal muscle. From such analytical data the total body content of cytochrome *c* can be reliably deduced (5, 6). The experimental procedures, complete thyroidectomy, thiouracil thyrotoxicosis, and hyperthyroidism induced by injection of thyroxine, were designed to bring out, if possible, by the opposite means of thyroxine deficiency and excess, any manifestations of relationship of the thyroid gland and cytochrome *c*. The results of the different experiments have been concordant, leading to the unequivocal conclusion that a positive correlation exists between thyroid function and tissue cytochrome *c* concentration and content.

Methods

Adult albino rats, 170 to 270 gm. in weight, were used. Records were kept of the initial, preoperative (at time of liver lobectomy), and final body weights. The animals were maintained on the synthetic, high (31 per cent) protein diet, previously described (3). As in our earlier work (3-5), a 24 hour fasting period was allowed before the partial hepatectomy and before sacrifice of the animals at termination of the experiment. For details of technique, methods of calculation of "per cent restoration or regeneration (of liver)," and the surgical procedure of liver lobectomy reference may be made to previous communications (3, 5).

Experimental Procedures—Complete thyroidectomy was performed aseptically, under ether anesthesia. The gland was approached through a longitudinal incision over the trachea and removed from the sides of the trachea by careful, blunt dissection. In the rat the isthmus of the normal thyroid is either exceedingly fine or rudimentary. The gland has the appearance of two, small, brownish discrete bodies, which are removed separately, care being taken to retain the closely adjacent parathyroids.

In the thiouracil experiments, 50 mg. of the drug, incorporated in meas-

ured amounts of the dietary mixture, were given per day. The administration of thiouracil, at the above dosage for a period of about 2 months, resulted in an expected, but, nevertheless, striking enlargement of the thyroid tissue. In a normal control rat of 200 gm. the thyroid gland weighs approximately 0.02 gm. In thiouracil thyrotoxicosis, the hypertrophied glands were deep purple in color and weighed 0.15 to 0.19 gm., despite an accompanying reduction of 30 to 40 per cent in the body mass of these rats (Columns 2 and 3, Tables III and IV).

For the production of experimental hyperthyroidism, 1 mg. of crystalline, synthetic thyroxine (Roche-Organon), in weakly alkaline solution, was injected subcutaneously every other day through the loose skin of the back. Mildly alkaline solutions are well tolerated at this site, but not in regions where the skin is tight. There was a large loss in body weight of animals so treated for a period of 1 month (Columns 2 and 3, Table V and Table VI).

Care was taken to provide sufficient time to establish the desired experimental state. Thus, partial hepatectomy was performed 35 days after thyroidectomy, 45 days after daily dosage with thiouracil, and 14 days after inception of thyroxine injection, during which period seven successive doses of 1 mg. each of thyroxine were administered. Thiouracil treatment and thyroxine injection were continued in the respective procedures for 2 weeks after liver lobectomy, when the animals were sacrificed.

Analytical Procedures—The direct microspectrophotometric method of Rosenthal and Drabkin (12) was used to determine the concentration of cytochrome *c* in the excised liver and, at the termination of the experiment, in liver, kidney cortex, heart, and skeletal muscle. Upon aliquots of the tissues the wet weight to dry weight ratios were obtained as previously described (5). Because of their cytological significance and possible relation to protein synthesis in regenerating tissue, indicated in our earlier work (3), ribose nucleic acid (PNA) and desoxyribose nucleic acid (DNA) were also determined. An adaptation (3) of Schneider's method (13) was used for this purpose.

Results

The data presented in Tables I and II disclose that complete thyroidectomy has a pronounced influence on the concentration (and content) of cytochrome *c* and of PNA in all the tissues examined, but only a slight effect on the rate of liver restoration after partial hepatectomy. The decrease in the concentration of cytochrome *c* was of the order of 25 to 30 per cent in liver (Columns 7 and 10, Table I), kidney and heart (Table II), and still larger (47 per cent) in skeletal muscle (Table II). It may be estimated (5, 6) from these data that in the thyroidectomized animals the

total body content of cytochrome c was reduced by about 43 per cent. Inspection of the data indicates the high degree of consistency in individ-

TABLE I

Quantity of Liver Regeneration and New Cytochrome c in Liver after 14 Days of Restoration on High (31 Per Cent) Protein Diet, Following Excision of 68.4 Per Cent of Liver 35 Days after Thyroidectomy

Rat No.	Body weight		Liver excised		Weight of original total liver	Amount of re-maining liver	Cytochrome c in remaining liver†		Final total liver		New or restored liver tissue	Cytochrome c in final liver‡		New cytochrome c in restored liver§			
	Preoperative	Final	Amount	W:D*			(7)	(8)	(10)	(11)							
												Per gm. tissue, wet weight	Total	Amount	W:D	Per gm. tissue, wet weight	Total
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)							
	gm.	gm.	gm.		gm.	gm.	γ	γ	gm.		per cent	γ	γ	γ	per cent		
3	182	182	3.54	3.34	5.18	1.64	136	223	4.09	3.38	69.2	192	785	562	71.6		
5	173	171	3.26	3.42	4.77	1.51	149										
6	192	192	3.70	3.12	5.41	1.71	133	227	4.11	3.26	64.8	176	723	496	68.6		
8	210	216	4.19	3.06	6.13	1.94	147	285	4.88	3.38	70.2	185	903	618	68.4		
9	200	190	4.07	3.05	5.95	1.88	123	231	4.18	3.24	56.5	170	710	479	67.5		
Mean ± s.e.				3.20			138			3.32	65.2	181	780	539	69.0		
(Rats 3-9)				±0.07			±5			±0.04		±5					
Rate of appearance of new cytochrome c per day.....														39	4.9		
Mean ± s.e.				3.32			178			3.31	73.9	210	1325	920	67.6		
(control rats) ¶				±0.05			±4			±0.04		±5					
Rate of appearance of new cytochrome c per day.....														66	4.8		

* Wet weight to dry weight ratios.

† The values for the concentration of cytochrome c per gm. of wet weight of the remaining liver were obtained from the analyses of excised liver. The values for total cytochrome c were calculated by multiplying the concentration of the pigment (Column 7) by the corresponding weights of remaining liver tissue (Column 6).

‡ The values for total cytochrome c were calculated by multiplying the concentration of the pigment (Column 10) by the corresponding weights of final total liver (Column 8).

§ Total cytochrome c in final liver (Column 10) minus the corresponding values for total cytochrome c in the remaining liver (Column 7).

|| Standard error = $\sqrt{\Sigma d^2/n(n-1)}$.

¶ From data of Drabkin (3).

ual animals. The significance of the difference between the means of the control and experimental (thyroidectomized) rats was tested objectively

by applying Fisher's criterion of t (14). Fisher's table of t was entered by means of the equations

$$t = (m_1 - m_2) / \sqrt{s.e._1^2 + s.e._2^2} \quad \text{and} \quad n = (n_1 - 1) + (n_2 - 1)$$

where m_1 and m_2 represent the two means, $s.e._1$ and $s.e._2$ the two standard errors (see the foot-note to Table I), and n_1 and n_2 are the number of observations from which the respective means are obtained. According to this test, the results in the thyroidectomized (Tables I and II) and also in the thiouracil- and thyroxine-treated animals (Tables III to VI) were

TABLE II

Concentration (C) of Cytochrome c and PNA and DNA in Liver, and Concentration of Cytochrome c in Other Tissues of Rats Subjected to Liver Lobectomy 35 Days after Thyroidectomy

The values are per gm. wet weight of tissue.

Rat No.	Liver at operation				Liver after 14 days regeneration				Heart		Kidney cortex		Skeletal muscle	
	C	W:D*	PNA	DNA	C	W:D	PNA	DNA	C	W:D	C	W:D	C	W:D
	γ		mg.	mg.	γ		mg.	mg.	γ		γ		γ	
3	136	3.34	6.95	2.73	192	3.38	8.05	2.78	329	4.02	225	4.43	58	3.65
5	149	3.42	7.90	2.67										
6	133	3.12	7.65	2.99	176	3.26	9.30	2.89	286	4.13	255	4.62	64	3.58
8	147	3.06	7.40	2.79	185	3.38	8.45	2.67	334	4.32	277	4.25	52	3.91
9	123	3.05	6.05	2.84	170	3.24	7.60	2.84			234	4.46	57	3.95
Mean \pm s.e. * (Rats 3-9)	138 ± 5	3.20 ± 0.07	7.19 ± 0.10	2.80 ± 0.05	181 ± 5	3.32 ± 0.04	8.35 ± 0.11	2.80 ± 0.06	316 ± 16	4.16 ± 0.08	248 ± 12	4.44 ± 0.08	58 ± 3	3.77 ± 0.09
Mean \pm s.e. (controls)†	178 ± 4	3.32 ± 0.05	8.50 ± 0.25	2.46 ± 0.02	210 ± 5	3.31 ± 0.04	6.96 ± 0.24	3.53 ± 0.04	449 ± 41	4.42 ± 0.12	346 ± 23	4.08 ± 0.09	110 ± 5	4.03 ± 0.02

* See the foot-notes to Table I.

† Values for cytochrome c, PNA, and DNA in liver from data of Drabkin (3); values for cytochrome c in heart, kidney, and skeletal muscle from data of Crandall and Drabkin (5).

uniformly of very high statistical significance. The vast majority of comparisons had values of t in the range of 4 to 8, with a corresponding probability, P (obtained from the table of t (14)), of less than 0.01 that the difference may be attributable to chance.

The increase in concentration of cytochrome c in regenerating liver above the preoperative liver level, consistently observed in our earlier experiments (3, 5), was also found following thyroidectomy (compare values in Columns 7 and 10, Table I), despite the reduction in quantity of this chromoprotein. It may also be seen that liver regeneration was only moderately (about 12 per cent), perhaps not significantly, decreased in

comparison with the normal controls (Column 9, Table I), and that the percentage of new cytochrome *c* in restored liver and the rate of its appearance (Column 11, Table I) were similar in the thyroidectomized and normal rats. The striking parallelism of change in PNA and cytochrome *c*, reported previously (3), is evident in the present data which show a drop in liver PNA after the removal of the thyroid and an increase in this

TABLE III
Quantity of Liver Regeneration and New Cytochrome c in Liver after 14 Days of Restoration, Following Excision of 68.4 Per Cent of Liver of Rats Treated with Thiouracil

The rats received 50 mg. of thiouracil per day in a measured amount of high protein diet for 45 days before and 13 days after operation.

Rat No.	Body weight		Liver excised		Weight of original total liver	Amount of re-remaining liver	Cytochrome <i>c</i> in remaining liver*		Final total liver		New or restored liver tissue	Cytochrome <i>c</i> in final liver*		New cytochrome <i>c</i> in restored liver*		
	Preoperative	Final	Amount	<i>W:D</i> *			(7)		(8)			(9)	(10)		Total	New cytochrome <i>c</i> in restored liver*
							Per gm. tissue, wet weight	Total	Amount	<i>W:D</i>			Per gm. tissue, wet weight	Total		
(1)	(2)	(3)	(4)	(5)	(6)	Per gm. tissue, wet weight	Total	Amount	<i>W:D</i>	(9)	Per gm. tissue, wet weight	Total	(11)			
	gm.	gm.	gm.		gm.	gm.	γ	γ	gm.		per cent	γ	γ	γ	per cent	
10	215	164	3.81	3.27	5.57	1.76	156	275	5.54	3.33	99.2	160	896	621	69.3	
12	200	150	3.53	3.33	5.18	1.64	141	231	4.12	3.40	70.3	151	622	391	62.8	
14	216	156	3.49	3.39	5.10	1.61	157	253	5.00	3.37	97.1	174	870	617	70.9	
16	226	162	3.76	3.47	5.50	1.74	149	259	4.31	3.49	68.3	175	754	495	65.6	
20	221	148	3.80	3.45	5.56	1.76	148	260	4.59	3.35	74.4	165	757	497	65.6	
21	270	171	4.05	3.44	5.93	1.88	140	263	4.53	3.43	69.7	167	757	494	65.2	
Mean ± S.E.*				3.39			149			3.40	79.8	165	776	519	66.6	
(Rats 10-21)				±0.04			±3			±0.02		±3				
Rate of appearance of new cytochrome <i>c</i> per day														37	4.8	
Mean ± S.E.				3.32			178			3.31	73.9	210	1325	920	67.6	
(control rats)*				±0.05			±4			±0.04		±5				
Rate of appearance of new cytochrome <i>c</i> per day														66	4.8	

* For calculations and symbols see the foot-notes to Table I.

cytoplasmic nucleic acid component during liver restoration (Table II). There was no significant change in the water content of tissues after thyroidectomy (Tables I and II), and the body weight was not altered markedly (Columns 2 and 3, Table I).

The results of thiouracil administration are reported in Tables III and IV. It may be seen that thiouracil thyrotoxicosis is attended by changes

TABLE IV

Concentration (C) of Cytochrome c and PNA and DNA in Liver, and Concentration of Cytochrome c in Other Tissues of Rats Treated with Thiouracil

The values are per gm. of wet weight of tissue. Rats 10 to 21 received 50 mg. of thiouracil per day. Rats 22 to 26 received 50 mg. of thiouracil and 5 mg. of folic acid per day. Rats 27 to 31 received 50 mg. of thiouracil and 10 mg. of folic acid per day.

Rat No.	Body weight		Liver				Heart		Kidney cortex		Skeletal muscle	
	Initial*	Final	C	W:D†	PNA	DNA	C	W:D	C	W:D	C	W:D
	gm.	gm.	γ		mg.	mg.	γ		γ		γ	
10	215	164	156	3.27	8.85	2.67						
10r†		164	160	3.33	9.20	2.67	332	4.17	251	3.87	67	3.84
11	206	170	138	3.20	7.80	2.73	351	3.72	264	4.02	55	3.92
12	200	150	141	3.33	7.85	2.73						
12r		150	151	3.40	8.55	2.73	305	4.24	231	4.18	49	4.01
13	189	147	145	3.27	8.05	2.79	338	4.31	242	4.35	51	4.34
14	216	156	157	3.39	8.75	2.73						
14r		156	174	3.37	11.35	2.84	342	3.81	265	4.07	60	4.10
15	188	140	152	3.29	8.20	2.99	318	4.38	264	4.01	58	4.05
16	226	162	149	3.47	7.95	2.96						
16r		162	175	3.49	10.35	2.67	336	4.35	251	4.37	56	3.72
17	236	165	137	3.36	8.15	2.27	331	4.03	234	4.15	59	3.91
18	235	154	129	3.39	7.35	2.55	321	4.51	226	4.57	61	4.16
19	274	183	152	3.57	7.40	2.44	343	4.83	273	4.32	46	4.55
20	221	148	148	3.45	7.35	2.67						
20r		148	165	3.35	9.45	2.56	344	4.40	252	4.27	46	3.94
21	270	171	140	3.44	7.55	2.73						
21r		171	167	3.43	10.05	2.96	344	4.40	259	4.35	50	3.98
22	213	152	146	3.46	6.40	2.21	300	4.40	267	3.78	58	4.27
23	181	164	153	3.42	7.55	2.27	351	4.01	229	4.56	49	3.96
24	179	152	134	3.20	6.60	2.04	333	4.04	235	4.32	65	4.29
25	183	155	127	3.43	6.95	2.32	329	4.08	242	4.22	71	3.88
26	183	157	157	3.33	6.70	2.49	342	4.12	251	4.06	43	4.26
27	174	146	128	3.32	6.90	2.32	337	3.93	234	4.39	52	4.72
28	182	146	157	3.32	6.95	2.61	300	4.45	224	4.61	64	4.31
29	201	145	136	3.24	7.40	2.32	370	4.19	247	4.20	48	4.16
30	184	143	145	3.46	6.40	2.04	326	3.70	253	4.14	57	4.01
31	192	156	139	3.44	6.30	2.27	320	3.92	266	4.06	52	4.27
Mean ± s.e.†			144	3.37	7.43	2.51	332	4.16	247	4.24	56	4.19
(Rats 10-31)			±2	±0.02	±0.50	±0.06	±1	±0.08	±4	±0.06	±2	±0.06
Mean ± s.e.			165	3.40	9.83	2.73	334	4.23	251	4.19	55	3.93
(Rats 10r-21r)			±3	±0.02	±0.40	±0.05	±6	±0.09	±5	±0.08	±3	±0.05

TABLE IV—*Concluded*

Rat No.	Body weight		Liver				Heart		Kidney cortex		Skeletal muscle	
	Initial*	Final	C	W:D†	PNA	DNA	C	W:D	C	W:D	C	W:D
			γ		mg.	mg.	γ		γ		γ	
Mean \pm s.e.			178	3.32	8.50	2.46	447	4.34	352	4.07	98	3.89
(Controls, preoperative)†			± 4	± 0.05	± 0.25	± 0.02	± 16	± 0.05	± 21	± 0.05	± 6	± 0.04
Mean \pm s.e.			210	3.31	6.96	3.53	449	4.42	346	4.08	110	4.03
(controls, postoperative)†			± 5	± 0.04	± 0.24	± 0.04	± 41	± 0.12	± 23	± 0.09	± 5	± 0.02

* Or preoperative for animals designated by *r*.

† For symbols and explanations see the foot-notes to Tables I and II.

‡ *r*, results after 14 days of liver regeneration.

in cytochrome *c* and PNA which are essentially similar in direction and magnitude to those obtained with complete thyroidectomy. As has already been stated, the differences between the means of the experimental animals and controls, tested by Fisher's method, are highly significant statistically. The decrease in cytochrome *c* was 16 to 30 per cent in liver (Columns 7 and 10, Table III), heart and kidney (Table IV), and 43 to 50 per cent in skeletal muscle (Table IV). The reduction in cytochrome *c* was of similar dimensions in the tissues of rats subjected to liver lobectomy to those in which this surgical procedure was omitted (compare data on Rats 10*r* to 21*r* with those on Rats 22 to 31, Table IV). From the changes in concentration it may be estimated (5, 6) that the total body content of cytochrome *c* was reduced by about 42 per cent in the thiouracil-treated animals.

Whereas a slight, though questionable, decrease in the rate of liver regeneration could be deduced from the data on the thyroidectomized rats, it is evident (Column 9, Table III) that no impairment whatever in this process is revealed by the data on the thiouracil-treated rats. Indeed, in two out of six animals, liver restoration appears to have been appreciably greater than in the controls. There is no satisfactory explanation for this finding, which was, at any rate, not constant. The drug thiouracil may exert a stimulatory effect on the regenerating liver. As in the thyroidectomy experiments, there was no significant change in the water content of the tissues after thiouracil administration. The only marked difference in the tabulated data of the thyroidectomized and thiouracil-treated groups is the appreciable loss in body weight of the latter (Columns 2 and 3, Tables III and IV).

It may be noted in Table IV that folic acid, at levels of 5 or 10 mg. per

day, was added to the diet of some of the rats receiving thiouracil. This was done to test the possibility that thiouracil might be antagonistic to folic acid, an idea suggested by certain resemblances in the structures of the two compounds. The experimental findings were entirely negative in this connection, since there was no evidence of any reversal of the thiouracil effect by the addition of the above quantities of folic acid.

TABLE V

Quantity of Liver Regeneration and New Cytochrome c in Liver after 14 Days of Restoration on High Protein Diet, Following Excision of 68.4 Per Cent of Liver of Rats Treated with Thyroxine

1 mg. of thyroxine was injected subcutaneously every other day for 2 weeks before and 2 weeks after operation.

Rat No.	Body weight		Liver excised		Weight of original total liver	Amount of remaining liver	Cytochrome c in remaining liver*		Final total liver		New or restored liver tissue	Cytochrome c in final liver*		New cytochrome c in restored liver*	
	Initial	Final	Amount	W:D*			Per gm. tissue, wet weight	Total	Amount	W:D		Per gm. tissue, wet weight	Total	Per gm. tissue, wet weight	Total
	gm.	gm.	gm.		gm.	gm.	γ	γ	gm.		per cent	γ	γ	γ	per cent
54	211	138	4.73	3.78	6.93	2.20	228	502	4.81	3.50	55.2	209	1006	504	50.1
55	210	129	4.12	3.66	6.02	1.90	261	496	3.82	3.92	46.6	216	825	329	39.9
56	215	127	5.08	3.89	7.43	2.35	238	560	4.10	3.61	34.5	202	829	269	32.5
57	190	112	3.41	3.73	4.98	1.57	228	358	2.91	3.43	39.3	198	576	218	37.9
Mean ± s.e.* (Rats 54-57)				3.77 ±0.05			239 ±8			3.62 ±0.10	43.9	206 ±4	809	330	40.1
Rate of appearance of new cytochrome c per day														24	2.9
Mean ± s.e. (control rats)*				3.32 ±0.05			178 ±4			3.31 ±0.04	73.9	210 ±5	1325	920	67.6
Rate of appearance of new cytochrome c per day														66	4.8

* For calculations and symbols see the foot-notes to Table I.

The influence of the experimental, acute hyperthyroid state, induced by injection of thyroxine, on cytochrome c, PNA, and liver regeneration is illustrated by the data collected in Tables V and VI. At the outset it should be pointed out that different results were obtained on rats following liver lobectomy and on intact animals. In hyperthyroid rats not subjected to partial hepatectomy (Rats 36 to 41, Table VI) there was a

TABLE VI

Concentration (C) of Cytochrome c and PNA and DNA in Liver, and of Cytochrome c in Other Tissues of Rats Treated with Thyroxine

The values are per gm. wet weight of tissue. 1 mg. of thyroxine was injected subcutaneously every other day for 2 weeks in Rats 36, 37, and 42 to 45, for 4 weeks in Rats 38 to 41, and for 2 weeks before and 2 weeks after operation in Rats 54 to 57.

Rat No.	Body weight		Liver				Heart		Kidney cortex		Skeletal muscle	
	Initial*	Final	C	W:D†	PNA	DNA	C	W:D	C	W:D	C	W:D
	gm.	gm.	γ		mg.	mg.	γ		γ		γ	
36	185	149	234	3.35	10.50	2.94	636	4.06	426	4.54	134	3.89
37	171	138	236	3.41	11.30	2.39	630	4.10	401	4.60	143	3.85
38	186	142	242	3.53	11.00	2.62	603	4.15	449	4.35	122	4.40
39	208	175	244	3.29	11.05	2.40	618	4.10	417	4.90	155	3.84
40	190	139	273	3.35	11.25	2.61	650	4.13	404	4.86	116	4.66
41	199	157	254	3.25	11.40	2.67	587	4.23	435	4.95	130	4.32
42	224	133	221	3.66	11.00	2.62						
43	205	123	249	3.51	11.05	2.40						
44	215	136	232	3.46	11.25	2.61						
45	218	121	237	3.36	11.40	2.67						
54	211	138	228	3.78	11.05	2.39						
54r†	211	138	209	3.50	7.05	4.44	278	4.24	423	4.77	55	3.85
55	210	129	261	3.66	11.35	2.62						
55r	210	129	216	3.92	7.50	3.82	318	4.14	442	5.02	46	4.10
56	215	127	238	3.89	11.30	2.61						
56r	215	127	202	3.61	7.90	4.16	341	4.01	466	4.85	49	3.96
57	190	112	228	3.73	11.10	2.40						
57r	190	112	198	3.43	6.95	4.01	332	4.23	441	4.96	65	4.06
Mean ± s.e.† (Rats 36-57)			241 ±4	3.52 ±0.05	11.14 ±0.07	2.57 ±0.04	621 ±9	4.13 ±0.02	422 ±8	4.70 ±0.09	133 ±6	4.16 ±0.15
Mean ± s.e. (Rats 54r-57r)			206 ±4	3.62 ±0.10	7.35 ±0.21	4.11 ±0.13	317 ±14	4.16 ±0.05	443 ±9	4.90 ±0.06	54 ±4	3.99 ±0.06
Mean ± s.e. (controls, preopera- tive)†			178 ±4	3.32 ±0.05	8.50 ±0.25	2.46 ±0.02	447 ±16	4.34 ±0.05	352 ±21	4.07 ±0.05	98 ±6	3.89 ±0.05
Mean ± s.e. (controls, postopera- tive)†			210 ±5	3.31 ±0.04	6.96 ±0.24	3.53 ±0.04	449 ±41	4.42 ±0.12	346 ±23	4.08 ±0.09	110 ±5	4.03 ±0.02

* Or preoperative for animals designated by r.
† For symbols and explanations see the foot-notes to Table II.
‡ r, results after 14 days of liver regeneration.

statistically highly significant (see above) change in cytochrome c and PNA in the opposite direction from that observed following thyroidectomy or thiouracil administration. The cytochrome c was increased by

34 per cent in liver (Column 7, Table V) and 39, 20, and 36 per cent respectively in heart, kidneys, and skeletal muscle (Rats 36 to 41, Table VI). It is uncertain whether any special significance may be attached to the fact that in these experiments the most pronounced change occurred in the heart rather than in the skeletal muscle. From the concentration in the tissues it may be estimated (5, 6) that the total body content of cytochrome *c* in the intact hyperthyroid animals was approximately 35 per cent greater than in the normal controls and some 137 per cent greater than in the hypothyroid (thyroidectomized and thiouracil-treated animals). The striking parallelism of the concentrations of cytochrome *c* and PNA is again seen in the finding of the large increase in liver PNA from a mean of 8.50 to that of 11.14 mg. per gm. (Table VI).

The percentage of tissue restoration after liver lobectomy in the hyperthyroid rats was only 43.9 (Column 9, Table V), which is 41 per cent less than is usually obtained in normal animals under the same dietary conditions. This is the lowest degree of liver regeneration which we have thus far observed in any of our studies. The hyperthyroid state is apparently not conducive to the cellular proliferative process, despite the increased amounts of cytochrome *c* and PNA present preoperatively, which, on the basis of our earlier findings (3), might have been expected to favor regeneration (see "Discussion"). Consonant with the impaired liver regeneration in the partially hepatectomized, hyperthyroid animals are the following findings: (1) In place of the usual increase in cytochrome *c* during regeneration there was an actual, significant decrease (compare means in Columns 7 and 10, Table V). (2) The percentage of new cytochrome *c* in the restored liver and the rate of appearance of new pigment (Column 11, Table V) were markedly lower than in the controls.

Attention may now be directed to the apparently paradoxical results with respect to cytochrome *c* and PNA obtained in the hyperthyroid rats which were subjected to liver lobectomy 2 weeks after institution of thyroxine administration. In contrast with the finding of consistent increases in the cytochrome *c* concentration in all tissues of the intact thyroxine-treated animals (Rats 36 to 41, Table VI), in the partially hepatectomized hyperthyroid animals (Rats 54 to 57, Tables V and VI) only the kidneys had an increased cytochrome *c* concentration (Table VI). In these rats the concentration of the chromoprotein remained unchanged in liver (Column 10, Table V) and was actually significantly reduced in the heart and skeletal muscle (Table VI) below the values in the control rats. Since most of the cytochrome *c* is in skeletal muscle (5, 6), the low value for the concentration of the pigment in this tissue is largely responsible for an estimate of about 41 per cent reduction in the total body content of the chromoprotein in the animals subjected both to thyroxine injection and liver lobectomy.

DISCUSSION

The experimental results which have been reported lead us to propose *the existence of a functional relationship between the thyroid gland (and thyroxine) and cytochrome c in tissues*. Apparently conclusive support in favor of this thesis is furnished by the remarkable reduction in the tissue concentration and total body content of cytochrome *c* after either complete thyroidectomy or thiouracil intoxication and the equally striking increase in tissue concentration and body content of the chromoprotein in experimentally induced hyperthyroidism. The latter finding appears to shed light on earlier observations that tissues removed from animals treated with thyroid have a greater than normal rate of oxygen consumption (15) and higher succinoxidase and cytochrome oxidase activities (9), whereas the addition of thyroxine to normal tissues *in vitro* has no effect (15) or only a small effect (16). Moreover, Tissières' observation (11) that the administration of dinitrophenol, which raises the basal metabolic rate without increasing the oxygen consumption, has no effect on muscle cytochrome *c* suggests that the thyroxine effect is more specifically concerned with the oxygen utilization mechanism. The cytochrome *c*-thyroid relationship seems clearly to fit in with other correlations, such as that between the concentration of cytochrome *c* and the rate of oxygen consumption of tissues, and that of the total content of cytochrome *c* and body size, which have been reported in the preceding paper (6). Together, they suggest integrated relationships of probable fundamental biological importance, since they are of pertinence in the conjoined phenomena of *oxygen homeostasis* and cellular energy exchange.

Although at this stage it is preferable to stress merely the probable involvement of cytochrome *c* in the function of the thyroid gland in metabolism, it is tempting to carry deduction a step farther in a tentative postulate: *Thyroxine or thyroglobulin regulates the rate of oxygen consumption through the control of the tissue level of cytochrome c*. This proposal is compatible with the view that the concentration of cytochrome *c* is the limiting factor in the activity of the cytochrome oxidase system (6). It is offered as a first approximation in a rational explanation of the chemical control of the rate or intensity of cellular oxygen utilization, in which the thyroid hormone must have a major rôle. The view-point that hormones operate through the tissue enzymes has a precedent in the brilliant work of Cori and his colleagues (17) on the site of insulin action. Within the confines of the above hypothesis, the locus of action of thyroxine or thyroglobulin could be one of the components of the cytochrome biocatalytic chain. However, it must be made clear that our data do not reveal how the hormone influences the concentration of cytochrome *c*. The situa-

tion is far from simple. Indeed, as our findings on the parallel changes in PNA suggest, the hormonal effect need not be confined to cytochrome *c*, which, with its oxidase and PNA, is a constituent of the mitochondria (1, 18). Hence, the changes in cytochrome *c* may be only a part of far broader cytochemical alterations. Also, in the following paper (7) on the influence of adrenalectomy on cytochrome *c* it will be seen that a case has not been made out for an exclusive rôle of the thyroid in this connection.

Aside from the clue which they supply to a chemical mechanism of hormonal regulation of oxygen homeostasis, the changes in cytochrome *c* concentration are of interest in themselves. In the earlier literature, reviewed by Rosenthal *et al.* (19), the decrease in concentration of cytochrome *c* in neoplastic epithelium was recognized. The change was confined to the new growth, and its significance not understood (19). Generalized alterations in the tissue and body content of cytochrome *c*, such as the decrease and increase found here in experimental hypo- and hyperthyroidism respectively, have not been reported previously. It is suggested that these experimental findings may have a pertinent bearing upon the corresponding clinical disease states. Furthermore, positive means, thyroidectomy and thiouracil or thyroxine administration, are made available for modifying the tissue level of cytochrome *c*, which presumably cannot be altered by the injection of this chromoprotein (4). Whether such induced changes in cytochrome *c* concentration would have any therapeutic usefulness cannot be predicted.

The pronounced impairment of the regenerative process (in liver) in the hyperthyroid animals may be ascribed to the heightened metabolic demand of all the tissues, thus interfering with the necessary temporary diversion of materials to the proliferating tissue. Consonant with this is the relatively normal degree of liver restoration found in the hypothyroid states, despite the reduction in cytochrome *c*.

The paradoxical findings with respect to cytochrome *c* and PNA in hyperthyroid rats subjected to liver lobectomy possibly may be explained by the factor of toxicity. On the other hand, the partial removal of the liver had the effect of reversing the changes produced by thyroxine administration. This raises the question whether the liver itself may be involved in, or contribute to, the activity of the hormone, and illustrates some of the unresolved complexities of the problem.

Our thanks are due to Dr. Guy W. Clark of the Lederle Laboratories Division, American Cyanamid Company, for furnishing the folic acid used in some of the studies and to the Harrison Department of Surgical Research for the use of their facilities.

SUMMARY

1. Cytochrome *c*, PNA, and liver regeneration have been studied in three experimental states, complete thyroidectomy, thiouracil thyrotoxicosis, and acute hyperthyroidism induced by thyroxine injection.

2. Consistent and statistically highly significant reductions in the concentration of cytochrome *c* in tissues and in the tissue and total body content of the chromoprotein were found in the two hypothyroid states (thyroidectomy and thiouracil intoxication). While the decrease in cytochrome *c* was appreciable in all tissues examined, and, therefore, indicative of a generalized effect, it was especially pronounced (of the order of 50 per cent) in skeletal muscle. Equally significant increases in cytochrome *c* were demonstrated in the hyperthyroid state. The changes in liver PNA were strikingly parallel with those in cytochrome *c* under the various conditions.

3. Paradoxical results with reference to cytochrome *c* and PNA were obtained in hyperthyroid rats subjected to partial hepatectomy. In these animals liver regeneration was markedly impaired, whereas in the hypothyroid states it did not differ significantly from normal.

4. The findings appear to support unequivocally the deduction that there is a functional relationship between the thyroid gland and cytochrome *c* in tissues. This deduction is of importance as a first clue to a chemical mechanism involved in the hormonal control of oxygen homeostasis.

5. A further tentative hypothesis has been made: thyroxine or thyroglobulin regulates the rate of oxygen consumption through the agency of cytochrome *c*. This is only a working proposal, and the need of caution, at this stage, in the interpretation of the experimental data has been pointed out.

6. In some of the experiments the possibility that thiouracil may antagonize folic acid was tested. The addition of folic acid to the diet failed to modify the effect of the drug.

BIBLIOGRAPHY

1. Drabkin, D. L., *Federation Proc.*, **7**, 151, 483 (1948).
2. Drabkin, D. L., in Roughton, F. J. W., and Kendrew, J. C., *Haemoglobin*, London, 35 (1949).
3. Drabkin, D. L., *J. Biol. Chem.*, **171**, 395 (1947).
4. Drabkin, D. L., *J. Biol. Chem.*, **171**, 409 (1947).
5. Crandall, M. W., and Drabkin, D. L., *J. Biol. Chem.*, **166**, 653 (1946).
6. Drabkin, D. L., *J. Biol. Chem.*, **182**, 317 (1950).
7. Drabkin, D. L., *J. Biol. Chem.*, **182**, 351 (1950).
8. Tipton, S. R., *Endocrinology*, **34**, 181 (1944).

9. Tipton, S. R., Leath, M. J., Tipton, I. H., and Nixon, W. L., *Am. J. Physiol.*, **145**, 693 (1945-46).
10. Tissières, A., *Arch. internat. physiol.*, **54**, 305 (1946).
11. Tissières, A., *Arch. internat. physiol.*, **55**, 252 (1947).
12. Rosenthal, O., and Drabkin, D. L., *J. Biol. Chem.*, **149**, 437 (1943).
13. Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945); **164**, 747 (1946).
14. Fisher, R. A., *Statistical methods for research workers*, London, 7th edition, 177 (1938).
15. Rossiter, R. J., *J. Endocrinol.*, **2**, 165 (1940).
16. Rapport, D., Canzanelli, A., and Guild, R., *Endocrinology*, **36**, 260 (1946).
17. Cori, C. F., *Harvey Lectures*, **41**, 253 (1945-46). Colowick, S. P., Cori, G. T., and Slein, M. W., *J. Biol. Chem.*, **163**, 583 (1947).
18. Schneider, W. C., *J. Biol. Chem.*, **165**, 585 (1946). Hogeboom, G. H., Claude, A., and Hotchkiss, R. D., *J. Biol. Chem.*, **165**, 615 (1946). Schneider, W. C., Claude, A., and Hogeboom, G. H., *J. Biol. Chem.*, **172**, 451 (1948).
19. Rosenthal, O., and Drabkin, D. L., *J. Biol. Chem.*, **150**, 131 (1943); *Cancer Res.*, **4**, 437 (1944).

CYTOCHROME *c* METABOLISM AND LIVER REGENERATION. INFLUENCE OF ADRENALECTOMY*

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The possibility that there is a functional relationship between the thyroid gland and cytochrome *c* has been deduced from the remarkable effects, reported in the preceding communication (2), of thyroidectomy, thiouracil thyrotoxicosis, and thyroxine-induced hyperthyroidism upon the tissue and body content of the chromoprotein. For a clearer comprehension of these findings it was desirable to extend our study to the influence of other hormones. In this paper results will be presented of the effect of adrenalectomy on cytochrome *c*, ribose nucleic acid (PNA), and liver regeneration.

Conclusions drawn from earlier studies of the influence of the adrenals or adrenal cortical steroids (hormones) on the metabolism of tissues have not been concordant. Himwich *et al.* (3) found that adrenalectomy had no effect on the respiratory exchange of liver, kidney, and diaphragm, although the metabolism of the testicles and brain was depressed. The positive effect was interpreted by the above authors as non-specific and related to the low blood pressure. Later, Russell and Wilhelmi (4) reported that kidney slices from adrenalectomized animals had a definitely lower than normal rate of oxygen consumption, and that the normal rate was restored in kidney tissue from animals treated with desoxycorticosterone. These effects, however, could be demonstrated only with certain substrates, not with others (5). More recently, Tipton *et al.* (6) claimed that the succinoxidase and cytochrome oxidase activities of liver were reduced after adrenalectomy and restored in adrenalectomized rats given an active extract of the adrenal cortex. Tissières found a decrease in cytochrome *c* in rat skeletal muscle following adrenalectomy (7), but he was unable to demonstrate any significant effect on the concentration of the chromoprotein in the muscle tissue of normal animals

* This work was done under contract between the Office of Naval Research and the University of Pennsylvania. A preliminary report has been presented at the meetings of the Federation of American Societies for Experimental Biology at Detroit, April 18-22, 1949 (1).

treated with desoxycorticosterone acetate, nor in that of castrated rats injected with testosterone (8). The negative results with the cortical steroids, in contrast to those obtained with thyroxine (2, 7), led Tissières to conclude that the influence of adrenalectomy on cytochrome *c* probably was not that of specific action of adrenal hormones (8). The alteration in salt balance after adrenalectomy may in itself contribute to or be responsible for the changes in basal metabolism and oxygen consumption. Thus, Brownell and Hartman (9) reported normal basal metabolic rates for adrenalectomized dogs maintained with sodium salts, whereas Cohn *et al.* (10) found that the oxygen consumption of dogs with adrenal insufficiency was abnormally low, but could be restored to normal by the administration of sodium chloride. For an extensive review of the general metabolic aspects of adrenalectomy readers are referred to the recent article by Kendall (11).

Methods

The experiments were performed on albino rats, 170 to 200 gm. in weight. The animals were maintained on our high (31 per cent) protein diet described previously (12) for 2 weeks before surgery and for 13 days post-operatively. A 24 hour fasting period was allowed before operation and before the terminal sacrifice of the rats. The high protein character of the diet may be emphasized, since it is pertinent to the interpretation of some of our findings. Also, our synthetic ration afforded a liberal allowance of salts and vitamins (12). The salt mixture employed was slightly modified from that of Jones and Foster (13). It furnished an approximately equal amount (molar basis) of sodium and potassium, and was the main source of the inorganic constituents in the dietary. Bibliographic references to the technique of liver lobectomy and the analytical procedures for cytochrome *c*, PNA, etc., may be obtained in the preceding paper (2).

Bilateral (complete) adrenalectomy and partial hepatectomy were performed at the same time, aseptically, under ether anesthesia. Just before surgery 5 ml. of 5 per cent glucose in isotonic saline were administered intraperitoneally as a prophylactic measure designed to tide the animals over the operation and the immediate postoperative period. No supportive measures were employed during the survival period. These were avoided, since it is difficult to assess to what extent slight variations in maintenance, as the use of 1 per cent saline in place of drinking water during the early postoperative days (6) or during the entire survival period (14), may contribute to the differences in the findings on adrenalectomized animals, reported in the literature (*cf.* (9–11), and above).

Results

40 per cent of our adrenalectomized and partially hepatectomized rats failed to survive the full 14 day period. In a group of eight such ani-

TABLE I

Quantity of Liver Regeneration and New Cytochrome c in Liver after 14 Days of Restoration on High (31 Per Cent) Protein Diet, Following Excision of 68.4 Per Cent of Liver and Bilateral Adrenalectomy

Rat No.	Body weight		Liver excised		Weight of original total liver	Amount of remaining liver	Cytochrome c in remaining liver†		Final total liver		New or re-stored liver tissue	Cytochrome c in final liver‡		New cytochrome c in re-stored liver§	
	Initial	Final	Amount	W:D*			Per gm. tissue, wet weight	Total	Amount	W:D		Per gm. tissue, wet weight	Total		
	(2)	(3)													
(1)	gm.	gm.	gm.		gm.	gm.	γ	γ	gm.		per cent	γ	γ	γ	per cent
46	180	186	4.10	3.41	6.00	1.90	180	342	6.14	3.65	102.5	164	1007	665	66.0
49	194	180	4.39	3.62	6.42	2.03	173	352	5.41	3.88	77.0	170	920	568	61.8
51	168	172	3.77	3.54	5.52	1.75	159	278	5.23	3.51	92.3	197	1030	752	73.0
52	171	178	3.96	3.43	5.80	1.84	162	298	5.14	3.45	83.3	179	920	622	67.7
53	174	186	4.94	3.31	7.22	2.28	190	433	6.88	3.32	93.2	148	1018	585	57.5
Mean ± s.e.				3.46			173			3.56	89.7	172	979	638	65.2
(Rats 46-53)				±0.05			±6			±0.09		±8			
Rate of appearance of new cytochrome c per day.....														46	4.7
Mean ± s.e.				3.32			178			3.31	73.9	210	1325	920	67.6
(control rats)¶				±0.05			±4			±0.04		±5			
Rate of appearance of new cytochrome c per day.....														66	4.8

* W:D = wet weight to dry weight ratios.

† The values for the concentration of cytochrome c per gm. of wet weight of the remaining liver were obtained from the analyses of excised liver. The values for total cytochrome c were calculated by multiplying the concentration of the pigment (Column 7) by the corresponding weights of remaining liver tissue (Column 6).

‡ The values for total cytochrome c were calculated by multiplying the concentration of the pigment (Column 10) by the corresponding weights of final total liver (Column 8).

§ Total cytochrome c in final liver (Column 10) minus the corresponding values for total cytochrome c in the remaining liver (Column 7).

|| Standard error = $\sqrt{\Sigma d^2/n(n-1)}$.

¶ From data of Drabkin (12).

mals, two died within 24 hours after surgery; a third, with gross signs at autopsy of severe liver damage (fatty degeneration), died 7 days post-operatively. The five remaining animals, upon which the data collected

in Tables I and II were obtained, appeared to withstand the experimental procedure as well as could be expected under the circumstances. Objectively, signs of their relatively satisfactory condition were afforded by the maintenance of weight (Columns 2 and 3, Table I) and presumably of tissue hydration. The latter may be inferred from the insignificant alterations in the wet to dry weight ratios (compare means of *W:D*, Columns 4 and 8, Table I, and Columns 3 and 7, Table II).

A comparison of the mean values for cytochrome *c* in the experimental and control rats (Tables I and II) discloses that after adrenalectomy, as

TABLE II

Concentration (C) of Cytochrome c and PNA and DNA in Liver and Concentration of Cytochrome c in Other Tissues of Rats Subjected to Partial Hepatectomy and Complete Adrenalectomy

The values are per gm. wet weight of tissue.

Rat No. (1)	Liver at lobectomy				Liver after 14 days regeneration				Heart		Kidney cortex		Skeletal muscle	
	<i>C</i> (2)	<i>W:D</i> * (3)	PNA (4)	DNA (5)	<i>C</i> (6)	<i>W:D</i> (7)	PNA (8)	DNA (9)	<i>C</i> (10)	<i>W:D</i> (11)	<i>C</i> (12)	<i>W:D</i> (13)	<i>C</i> (14)	<i>W:D</i> (15)
	γ		mg.	mg.	γ		mg.	mg.	γ		γ		γ	
46	180	3.41	7.10	3.18	164	3.65	8.60	2.84	306	4.01	263	4.76	69	3.0
49	173	3.62	7.50	3.36	170	3.88	8.15	3.19	291	3.81	222	4.89	59	3.0
51	159	3.54	7.08	2.90	197	3.51	7.05	2.68	312	4.46	234	4.84	81	4.0
52	162	3.43	8.10	3.13	179	3.45	8.40	2.73	275	4.37	230	4.47	68	4.0
53	190	3.31	7.10	2.68	148	3.32	7.15	2.28	272	4.45	248	4.20	65	3.0
Mean \pm s.e. (Rats 46- 53)*	173 ± 6	3.46 ± 0.05	7.38 ± 0.20	3.05 ± 0.14	172 ± 8	3.56 ± 0.09	7.87 ± 0.32	2.74 ± 0.15	291 ± 8	4.22 ± 0.13	239 ± 7	4.63 ± 0.13	68 ± 4	4.0 ± 0.0
Mean \pm s.e. (controls)†	178 ± 4	3.32 ± 0.05	8.50 ± 0.25	2.46 ± 0.02	210 ± 5	3.31 ± 0.04	6.96 ± 0.24	3.53 ± 0.04	449 ± 41	4.42 ± 0.12	346 ± 23	4.08 ± 0.09	110 ± 5	4.0 ± 0.0

* See the foot-notes to Table I.

† Values for cytochrome *c*, PNA, and DNA in liver from data of Drabkin (12); values for cytochrome *c* in heart, kidney, and skeletal muscle from data of Crandall and Drabkin (13).

after thyriodectomy or thiouracil intoxication (2), there was a consistent and marked decrease in the concentration of the chromoprotein in all the tissues examined. Cytochrome *c* was reduced by 18 per cent in liver (Column 10, Table I) and by 35, 31, and 38 per cent in heart, kidney, and skeletal muscle respectively (Table II). Statistically, these changes are highly significant. By applying Fisher's criterion of *t* (2, 16) as an objective test of the significance of the differences between the means of the adrenalectomized and control animals, *t* values of 3.8 to 6.6 were obtained. Such values correspond with a probability, *P*, of less than 0.01 that the results may be ascribed to chance. From the concentrations of the pig-

ment in the tissues it was estimated (2, 15) that the total body content of cytochrome *c* had been reduced by about 36 per cent after adrenalectomy. This may be compared with a reduction of 42 to 43 per cent in the body content of the chromoprotein found by us in experimental hypothyroid states (2).

The usual rise, above the preoperative level, in the concentration of cytochrome *c* in liver during restoration (12), which was still observed in thyroid deficiency (2), is absent in the adrenalectomized rats (compare Columns 7 and 10, Table I). Perhaps related to this is the lack of change in PNA in the regenerating liver (Table II). On the basis of deductions from our earlier results in normal animals (12), these findings would be consistent with some impairment of the proliferative process. Evidently, this is not the case in the present experiments. An unexpected feature in these adrenalectomized rats is the consistent and significantly greater than normal degree of liver regeneration (Column 9, Table I; the mean values of 89.7 and 73.9 per cent differ by 21 per cent).

DISCUSSION

The confinement of the investigation to a single tissue, as in the work of Tissières (7, 8), was regarded as inadequate for an establishment of possible hormonal influences upon cytochrome *c*, a constituent of all cells. Generalized effects on the chromoprotein, suggestive of hormonal involvement, have now been demonstrated by us for the thyroid gland (preceding report (2)) and for the adrenals (present work). The finding that cytochrome *c* is depleted after adrenalectomy appears to have a pertinent bearing upon occasional earlier observations of a reduction in basal metabolic rate or in oxygen consumption in experimental adrenal insufficiency (4, 6, 10). It would be of interest to know whether comparable reductions in cytochrome *c* occur in humans with Addison's disease. If our results on the adrenalectomized animals are considered objectively, it must be concluded that, within the scope of existing information, the hormonal effect of the thyroid gland on cytochrome *c* (2) cannot be regarded as exclusive. Thyroid-adrenal interrelationships could be operative here. Nevertheless, the action of the thyroid hormone relative to the hemin protein is probably more direct and specific than that of an adrenal factor or factors. This view-point is favored by the clearly consistent and reproducible effects of thyroidectomy or thyroxine administration on the rate of oxygen consumption, in contrast to the irregular or equivocal findings in this respect after adrenalectomy (3-6, 9-11). Also, as already mentioned, the reduction in oxygen consumption sometimes found in adrenal insufficiency has been interpreted as an indirect effect related to the disturbed salt metabolism (10). Whether the observed

changes in cytochrome *c* after adrenalectomy are expressions of secondary rather than primary effects remains a moot question, owing to the complex nature of adrenal hormonal control.

The greater than normal liver regeneration found in our partially hepatectomized and adrenalectomized rats seems puzzling and is deserving of comment. While these studies were in progress, Friedgood, Vars, and Zerbe (of the Harrison Department of Surgical Research) investigated independently the nitrogen metabolism and liver restoration in rats with similar operations. In a brief abstract of their work (14), it is reported that liver regeneration was reduced in such animals, whether they were protein-depleted or protein-fed before operation. From the complete data (to be published) of Vars and his colleagues, it may be deduced that the level of protein fed may be an important determinant of the degree of liver restoration in the adrenalectomized animals. Hence, the unusual liver regeneration in our rats is probably related to the high (31 per cent) protein diet on which they were maintained pre- and postoperatively. The finding appears to support the view that in adrenalectomy under proper conditions the utilization of exogenous and endogenous protein goes on as effectively as in the normal (17). It is also an excellent illustration of the influence of maintenance factors upon the results in adrenalectomy (11).

We are indebted to Dr. Charles E. Friedgood, Fellow of the Harrison Department of Surgical Research, for performing the necessary surgery.

SUMMARY

In a continuation of the investigation of hormonal influences on cytochrome *c* and liver regeneration, the effect of bilateral adrenalectomy has been studied in rats maintained on a high (31 per cent) protein diet.

After complete adrenalectomy and partial hepatectomy there was a consistent and statistically significant reduction in the cytochrome *c* concentration and content of all tissues examined. These findings are very similar to those previously obtained after thyroidectomy (2).

The possibility that thyroid-adrenal interaction may be operative in the control of the tissue level of cytochrome *c* has been considered in the interpretation of the findings, although the thyroid is believed to be more directly or specifically involved.

Despite the depletion of cytochrome *c*, the regeneration of liver in our partially hepatectomized and adrenalectomized animals was appreciably greater than in normal controls. This unexpected finding has been attributed to the effect of the high protein maintenance diet.

BIBLIOGRAPHY

1. Drabkin, D. L., *Federation Proc.*, **8**, 195 (1949).
2. Drabkin, D. L., *J. Biol. Chem.*, **182**, 335 (1950).
3. Himwich, H. E., Fazekas, J. F., Barker, S. B., and Hurlburt, M. H., *Am. J. Physiol.*, **110**, 348 (1934-35).
4. Russell, J. A., and Wilhelmi, A. E., *J. Biol. Chem.*, **137**, 713 (1941).
5. Russell, J. A., and Wilhelmi, A. E., *J. Biol. Chem.*, **140**, 747 (1941).
6. Tipton, S. R., Leath, M. J., Tipton, I. H., and Nixon, W. L., *Am. J. Physiol.*, **145**, 693 (1945-46).
7. Tissières, A., *Arch. internat. physiol.*, **54**, 305 (1946).
8. Tissières, A., *Arch. internat. physiol.*, **55**, 252 (1947).
9. Brownell, K. A., and Hartman, F. A., *Endocrinology*, **29**, 430 (1941).
10. Cohn, C., and Soskin, S., *Am. J. Physiol.*, **139**, 80 (1943).
11. Kendall, E. C., in Harris, R. S., and Thimann, K. V., *Vitamins and hormones*. New York, **6**, 277 (1948).
12. Drabkin, D. L., *J. Biol. Chem.*, **171**, 395 (1947).
13. Jones, J. H., and Foster, C., *J. Nutr.*, **24**, 245 (1942).
14. Friedgood, C. E., Vars, H. M., and Zerbe, J. W., *Federation Proc.*, **8**, 200 (1949).
15. Crandall, M. W., and Drabkin, D. L., *J. Biol. Chem.*, **166**, 653 (1946).
16. Fisher, R. A., *Statistical methods for research workers*, London, 7th edition, 177 (1938).
17. Long, C. N. H., Katzin, B., and Fry, E. G., *Endocrinology*, **26**, 309 (1940). Anderson, E., *Essays in biology in honor of Herbert M. Evans*, Berkeley, **35** (1943). Ingle, D. J., and Oberle, E. A., *Am. J. Physiol.*, **147**, 222 (1946).

PRELIMINARY STUDIES ON THE AMINO ACID CONTENT OF A HIGH POTENCY PREPARATION OF THE OXYTOMIC HORMONE OF THE POSTERIOR LOBE OF THE PITUITARY GLAND*

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The preparation by the counter-current technique of high potency oxytomic material from the posterior lobe of the pituitary gland has recently been reported from this laboratory (1). The characteristics of the distribution curve suggested that this material was very nearly pure or that, if any impurity was present, it had a distribution coefficient almost identical with that of the oxytomic factor. The potency of the preparation was about 850 units per mg. when assayed by the chicken blood pressure method of Coon (2).

A number of investigations have been carried out on the amino acid content of oxytomic material. Several years ago du Vigneaud and collaborators (3) examined a series of preparations of pitocin of varying potencies for the presence of cystine. The most potent sample of pitocin examined (500 units of oxytomic activity per mg.) possessed a cystine content of 8.96 per cent as determined by the Sullivan method (4). Somewhat later Stehle and Fraser (5), also using the Sullivan method, found 8.92 per cent of cystine in an oxytomic preparation which had a potency of 250 units per mg. More recently Potts and Gallagher (6) found that their material, which had a potency of 700 units per mg., contained 18.3 per cent of cystine as determined by the Sullivan method (7).

The tyrosine content of the material studied by du Vigneaud *et al.* (3) was found to be 14.3 per cent by the method of Folin and Looney (8), whereas Stehle and Fraser, employing the method of Folin and Denis (9), found 10.7 per cent of tyrosine in their material. Potts and Gallagher, using the procedure of Arnow (10), found 14.2 per cent of tyrosine in their preparation of 700 units per mg.

The preparation of Stehle and Fraser also contained 6.1 per cent of arginine as determined by the method of Jorpes and Thorén (11), but Potts and Gallagher reported that their material, which was nearly 3 times as

*The authors wish to express their appreciation to the Lederle Laboratories Division, American Cyanamid Company, for research grants which have aided greatly in this work.

potent, contained less than 0.8 per cent of arginine. Stehle and Fraser and Stehle and Trister (12) also examined their material of 250 units per mg. for other amino acids and reported the presence of proline and leucine as well as cystine, tyrosine, and arginine. They also reported the absence of isoleucine, histidine, glutamic acid, hydroxyproline, glycine, and phenylalanine. No tests were made to determine whether alanine, aspartic acid, hydroxyglutamic acid, lysine, methionine, serine, threonine, or valine was or was not present. A summary of these and other studies is given in a review by Irving and du Vigneaud (13).

The development by Moore and Stein of an excellent method for the separation of small quantities of amino acids by chromatography on starch columns and their determination by a quantitative ninhydrin reaction (14-17) has allowed us to determine the amino acid composition of the high potency material prepared by Livermore and du Vigneaud (1).

By use of three separate columns, the method allows for the separation of most of the common constituents of protein hydrolysates. Leucine, isoleucine, methionine, tyrosine, and valine are separated by use of a column with an *n*-butanol-benzyl alcohol-water solvent system. Proline, threonine, aspartic acid, serine, glycine, ammonia, arginine, lysine, histidine, and cystine can be separated with 1:2:1 *n*-butanol-*n*-propanol-0.1 N HCl followed by 2:1 *n*-propanol-0.5 N HCl as solvents for eluting the amino acids from the column. Glutamic acid and alanine emerge together under these conditions, but can be separated by the use of a solvent mixture of 2:1:1 *tert*-butanol-*sec*-butanol-0.1 N HCl. As reported by Moore and Stein, the above amino acids can be quantitatively recovered with an accuracy of ± 3 per cent (16).

The present paper reports the results of the application of the starch column chromatographic method to the determination of the amino acid composition of high potency oxytomic material prepared by counter-current distribution.

EXPERIMENTAL

Starting Material and Hydrolysis—The material was combined from Tubes 15 and 16 containing the peak of the activity of the preparation described by Livermore and du Vigneaud ((1), Fig. 4) and lyophilized. The assay value was approximately 800 oxytomic units per mg. when determined by the method of Coon (2). 12.5 mg. were hydrolyzed, as recommended by Stein and Moore (14), for 20 hours in 200 volumes of 5.8 N HCl which had been distilled twice in glass. There was no visible humin precipitate; the solution, however, was yellow following hydrolysis. The sample was then evaporated to dryness three times *in vacuo*, and made up to a volume of 10 ml. with water. For analysis on the column aliquots of this solution

were delivered from a calibrated pipette to a small beaker, and were evaporated to dryness in a vacuum desiccator. An aliquot was dissolved in 0.5 ml. of the solvent to be used for separating the amino acids on the column, and then placed on the column by the method described by Moore and Stein (16). No moisture or ash determinations were made on the unhydrolyzed material.

Amino Acid Analysis—The preparation of the starch columns, addition of the sample to the column, and collection and analysis of the effluent fractions were all performed in as identical a fashion as possible with that described by Moore and Stein (14–16). The use for the elution of amino acids from a column of a 1:2:1 *n*-butanol-*n*-propanol-0.1 *N* HCl solvent followed by 2:1 *n*-propyl alcohol-0.5 *N* HCl gives a general picture of the amino acid composition of a hydrolysate. Fig. 1, *a* presents an example for the oxytocic material. An amount corresponding to 1.74 mg. of unhydrolyzed substance was placed on the column. It can be seen that peaks appear only at the positions that are ascribed (16) to leucine plus isoleucine, tyrosine plus valine plus methionine, proline, glutamic acid plus alanine, aspartic acid, glycine, ammonia, and cystine. The slight bump at ml. 9 of effluent is the colored material which is formed during hydrolysis. It does not react with ninhydrin. The presence of a symmetrical peak in the tyrosine, valine, and methionine range indicates a single amino acid in this peak rather than two or three. This peak in other chromatograms has given a positive qualitative test for tyrosine by use of the Folin-Ciocalteu phenol reagent (18). The rise before the beginning of the glycine peak at ml. 103 of effluent may indicate a small amount of serine.

Fig. 2 shows the results of running the hydrolysate through a column with 1:1:0.288 *n*-butanol-benzyl alcohol-water, a solvent which separates leucine, isoleucine, phenylalanine, tyrosine, valine, and methionine. Peaks are present for leucine, isoleucine, and tyrosine. Tubes from this tyrosine peak also gave a positive test for tyrosine. The small peak before the leucine indicates the presence of a trace of phenylalanine.

In order to determine whether the peak at the glutamic-alanine position (effluent ml. 61, Fig. 1, *a*) consisted of one or two components, another portion of the hydrolysate was run on a column with 2:1:1 *tert*-butanol-*sec*-butanol-0.1 *N* HCl. The pattern is given in Fig. 3 after the collection of a 52 ml. fore fraction. In this case 0.108 mg. of alanine was added to the sample before it was placed on the column. Calculation of the amount of amino acid in the alanine peak, as described in the following section, showed 0.112 mg. recovered. Thus the hydrolysate was completely free of alanine or contained only traces of it. From these data it would appear that the amino acids in the oxytocic material are leucine, isoleucine, tyrosine, proline, glutamic acid, aspartic acid, glycine, and cystine. After the above

information had been obtained by preliminary chromatograms, an artificial mixture corresponding approximately in composition to the pitocin hydrolysate was made up and run simultaneously with the chromatogram shown in

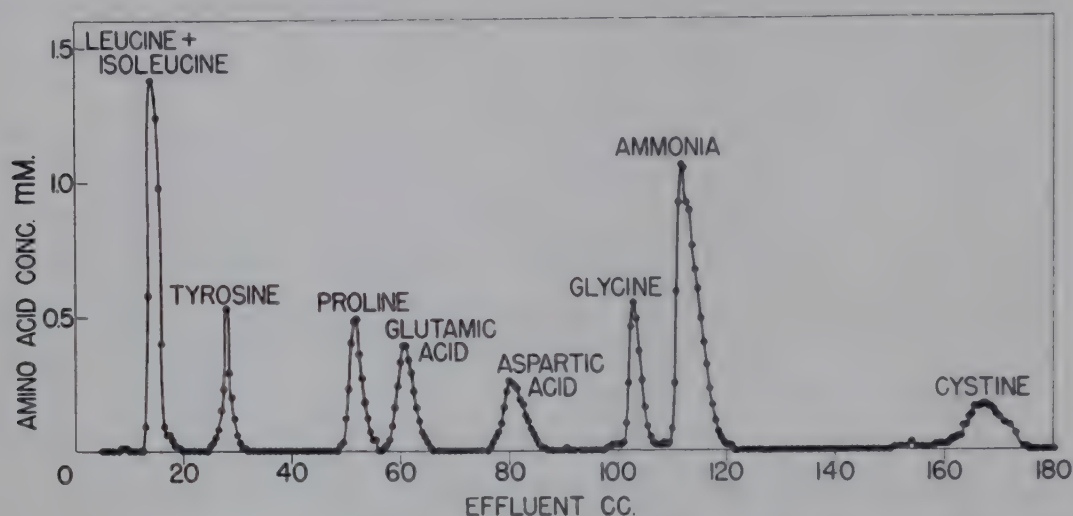


FIG. 1, *a*. Separation of amino acids from the hydrolysate of the oxytocic material. The solvents are 1:2:1 *n*-butanol-*n*-propanol-0.1 *N* HCl followed by 2:1 *n*-propanol-0.5 *N* HCl. The column contained 13.4 gm. of (anhydrous) starch; diameter, 0.9 cm.; column height, approximately 30 cm. The sample placed on the column is equivalent to 1.74 mg. of unhydrolyzed material.

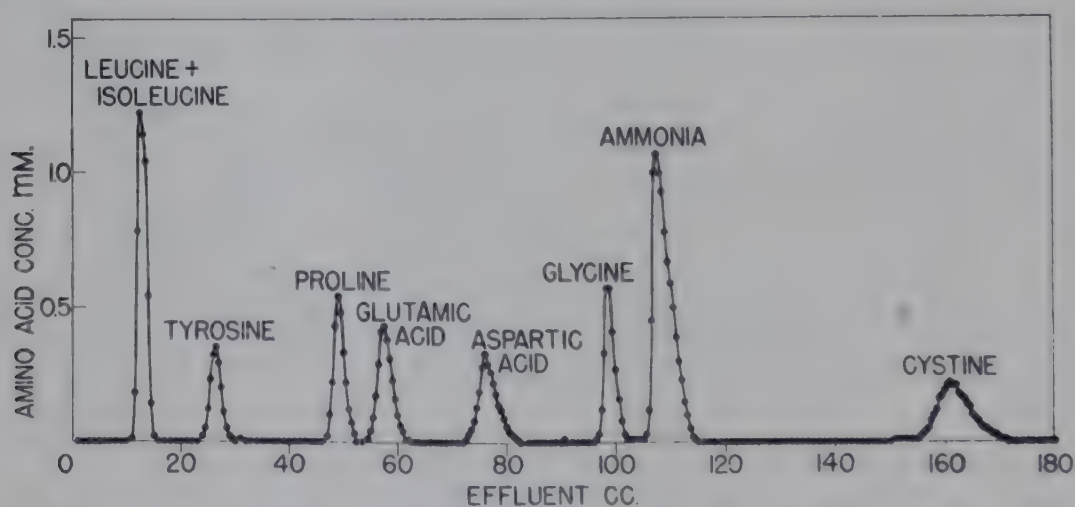


FIG. 1, *b*. Separation of amino acids from the artificial mixture simulating the amino acid composition of the hydrolysate of the oxytocic material. The solvents are 1:2:1 *n*-butanol-*n*-propanol-0.1 *N* HCl followed by 2:1 *n*-propanol-0.5 *N* HCl. The column contained 13.4 gm. of (anhydrous) starch; diameter, 0.9 cm.; column height, approximately 30 cm. The sample placed on the column contained 1.77 mg. of amino acids. The composition of the mixture is given in Table I.

Fig. 1, *a*.¹ The composition of the artificial mixture and the recovery of its amino acids are shown in Table I, and the pattern obtained from this

¹The amino acids used to make up the artificial mixture were commercial crystalline products with the exception of proline. The proline was a specially purified product kindly given to the authors by Dr. William H. Stein and Dr. Stanford Moore.

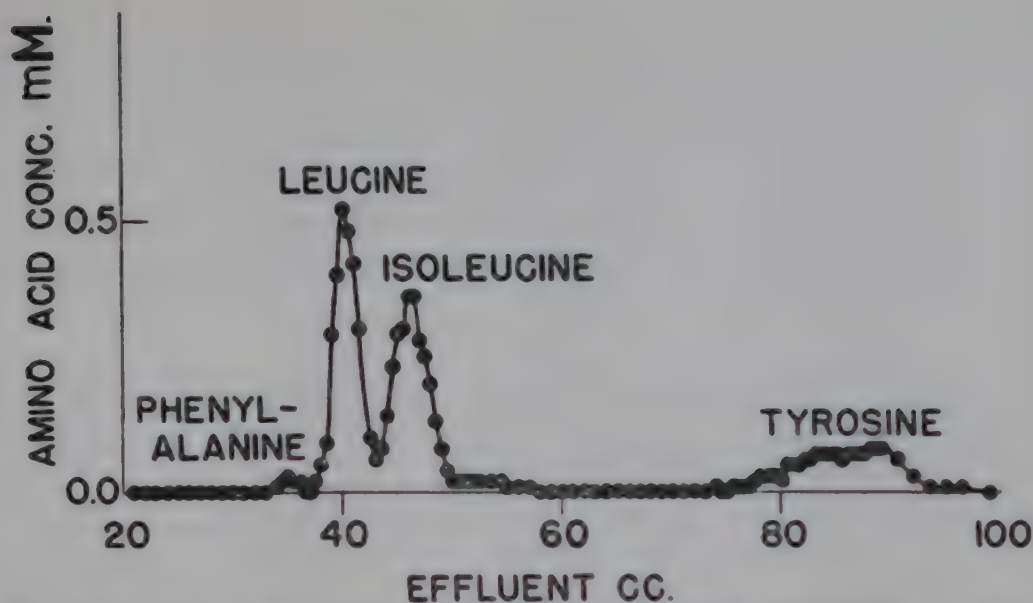


FIG. 2. Separation of leucine and isoleucine from the hydrolysate of the oxytocic material. The solvents are 1:1:0.288 *n*-butanol-benzyl alcohol-water (thiodiglycol added). The column contained 13.4 gm. of (anhydrous) starch; diameter, 0.9 cm.; column height, approximately 30 cm. The sample placed on the column is equivalent to 1.74 mg. of unhydrolyzed oxytocic material.

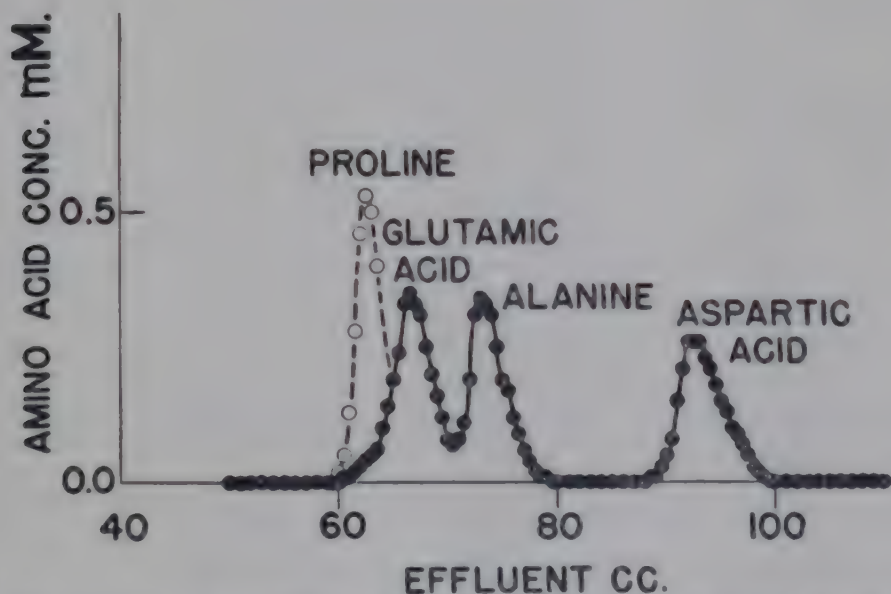


FIG. 3. Separation of glutamic acid and alanine from the hydrolysate of the oxytocic material with 0.108 mg. of alanine added. The solvent is 2:1:1 *tert*-butanol-*sec*-butanol-0.1 *N* HCl. The column contains 13.4 gm. of (anhydrous) starch; diameter, 0.9 cm.; height, approximately 30 cm. The sample placed on the column is equivalent to 1.74 mg. of unhydrolyzed oxytocic material plus 0.108 mg. of alanine.

mixture is given in Fig. 1, *b*. It can be seen that both samples, the hydrolysate of the natural product and the artificial mixture, give the same pattern of amino acid distribution.

Calculation of Results and Recovery of Amino Acids—Table I shows the recovery from the column of each amino acid found in the hydrolysate of

the oxytomic material, as determined by the quantitative ninhydrin method. The recovery is expressed as mg. of amino acid, amino acid residue, and nitrogen per mg. of unhydrolyzed oxytomic material. The molar ratios of the components to each other are also given, and the amounts of the artificial mixture placed on the column and their recovery are shown. The calculation of these values for the amino acids recovered from the col-

TABLE I
Amino Acid Composition of High Potency Oxytomic Material

The values for leucine and isoleucine are from the chromatogram given in Fig. 2; other values are from the chromatogram given in Fig. 1, *a*. The nitrogen content (one determination) was 0.149 mg. per mg. of unhydrolyzed oxytomic material.

Constituent	Amino acid per mg. oxytomic material*	Amino acid residue per mg. oxytomic material*	Nitrogen per mg. oxytomic material*	Molar ratio with leucine expressed as 1	Amino acids per mg. total artificial mixture placed on column	Amino acid per mg. total artificial mixture recovered from column
	mg.	mg.	mg.		mg.	mg.
Leucine.....	0.109†	0.094	0.0116	1.00	{ 0.211	{ 0.194
Isoleucine.....	0.107†	0.092	0.0114	0.98		
Tyrosine.....	0.108	0.097	0.0084	0.72	0.111	0.107
Proline.....	0.095	0.080	0.0115	0.99	0.082	0.083
Glutamic acid‡.....	0.131	0.115	0.0125	1.07	0.122	0.120
Aspartic ".....	0.114	0.096	0.0120	1.03	0.111	0.118
Glycine.....	0.063	0.045	0.0118	1.01	0.052	0.053
NH ₃	0.049	0.049	0.0400	3.46	0.135	0.129
Cystine.....	0.181	0.167	0.0211	1.01§	0.173	0.203
Total.....		0.835	0.140		0.997	1.01

* The values are expressed in relation to unhydrolyzed oxytomic material; moisture and ash content unknown.

† The value for the combined peak in the chromatogram of Fig. 1, *a* is 0.212.

‡ The glutamic acid and aspartic acid values are corrected for being 7 and 6 per cent low respectively (16), due to esterification in the solvent.

§ The cystine value has been corrected here for 10 per cent loss during hydrolysis (17).

|| Expressed as ammonium chloride.

umns was made by the method of Moore and Stein (15). The figure are plotted with ml. of effluent against amino acid concentration in mm per liter of leucine equivalents ((15), Table I). None of the peaks have been corrected for ninhydrin color yield relative to leucine except proline, which is read at 440 mμ instead of 570 mμ and has a color yield of only 0.27 at 440 mμ. The table of leucine equivalents, the correction factors for sample size

and solvent system, and the color yields of the amino acids relative to leucine are those given by Moore and Stein (15, 16). Determinations in this laboratory of these factors used in the calculation of results agreed with those of Moore and Stein (15), except that our values for the table of leucine equivalents were about 5 per cent higher. However, due to the good correlation in all but two cases between the amounts of amino acid of the artificial mixture which were added to the column and those recovered when the factors given by Moore and Stein (15) are employed, these factors were used for the calculation of the quantities of amino acids in the hydrolysate.

DISCUSSION

The identification of the peaks seems certain because of the identity of the patterns of the artificial mixture and the hydrolyzed oxytocic material. While the absolute position is not quite the same, the relative positions of the peaks in each curve correspond remarkably well (16). The results obtained, as shown in Table I, do not preclude the existence of a component or components which do not give a ninhydrin reaction. The 83.5 per cent recovery of amino acid residues becomes 87.3 per cent if corrections are made for the low tyrosine and low cystine values. Also the preparation could contain some acetic acid or acetate ion since it was lyophilized from an acetic acid solution. The moisture and ash contents of the sample are unknown. The recovery of nitrogen is better, with 94 per cent recovered. However, only enough sample was available for one micro-Kjeldahl determination, which was done on hydrolyzed material. The cystine value of 18.1 per cent (mg. of amino acid per mg. of unhydrolyzed protein) is in agreement with that obtained by Potts and Gallagher (6); however, 10 per cent of the cystine may have been destroyed during hydrolysis (17).

The low tyrosine value is difficult to explain in view of the fact that the molar ratios of all the other amino acids are 1:1 within experimental error. Subsequent work² indicates that part of the tyrosine was destroyed during hydrolysis. If one assumes a 1:1 ratio of tyrosine and corrects the ammonia value for this as well as for a 10 per cent destruction of cystine during hydrolysis, the ratio of ammonia to other components becomes 3.08:1. The fact that the compounds appear to be in equimolar ratio to each other with 3 moles of ammonia present indicates that the material prepared by counter-current distribution could well be a single chemical individual contaminated with only extremely small amounts of other amino acids or substances producing amino acids when hydrolyzed. For example, the amount of phenylalanine shown in the small peak in Fig. 2 is only 0.5 per cent of the weight of the starting material.

²Pierce, J. G., and du Vigneaud, V., unpublished work.

SUMMARY

The amino acid composition of high potency oxytocic material from the posterior pituitary prepared by counter-current distribution has been determined by chromatography on starch columns. With 12.5 mg. of material which had a potency of approximately 800 units per mg., it was found that the substance contained only leucine, isoleucine, tyrosine, proline, glutamic acid, aspartic acid, glycine, ammonia, and cystine. No more than extremely small traces of any other amino acids could be detected. Furthermore, within the error of the experiment, the molar ratios of these components were 1:1 with the exception of the ammonia and tyrosine. The low ratio of tyrosine to the other amino acids (0.72:1) may be due to partial destruction of the tyrosine during hydrolysis. The molar ratio of ammonia to the amino acids was 3.46:1. The nearly complete absence of other amino acids together with the 1:1 ratio of those present offers further evidence of the near homogeneity of material of 800 units per mg. which had been prepared by counter-current distribution.

The authors wish to express their deep appreciation to Dr. William H. Stein and Dr. Stanford Moore for their valuable advice and counsel in this work. The authors also wish to acknowledge the assistance of Mrs. Jacqueline Everett Parton and that of Dr. J. R. Rachele and Mrs. Josephine T. Marshall for the micro-Kjeldahl analysis.

BIBLIOGRAPHY

1. Livermore, A. H., and du Vigneaud, V., *J. Biol. Chem.*, **180**, 365 (1949).
2. Coon, J. M., *Arch. internat. pharmacod.*, **62**, 79 (1939).
3. du Vigneaud, V., Sealock, R. R., Sifferd, R. H., Kamm, O., and Grote, I. W., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **100**, p. xciv (1933).
4. Sullivan, M. X., *Pub. Health Rep., U. S. P. H. S.*, suppl. 78 (1929).
5. Stehle, R. L., and Fraser, A. M., *J. Pharmacol. and Exp. Therap.*, **55**, 136 (1935).
6. Potts, A. M., and Gallagher, T. F., *J. Biol. Chem.*, **143**, 561 (1942).
7. Sullivan, M. X., and Hess, W. C., *J. Biol. Chem.*, **117**, 423 (1937).
8. Folin, O., and Looney, J. M., *J. Biol. Chem.*, **51**, 421 (1922).
9. Folin, O., and Denis, W., *J. Biol. Chem.*, **12**, 245 (1912).
10. Arnow, L. E., *J. Biol. Chem.*, **118**, 531 (1937).
11. Jorpes, E., and Thorén, S., *Biochem. J.*, **26**, 1504 (1932).
12. Stehle, R. L., and Trister, S. M., *J. Pharmacol. and Exp. Therap.*, **65**, 343 (1939).
13. Irving, G. W., Jr., and du Vigneaud, V., *Ann. New York Acad. Sci.*, **43**, 273 (1943).
14. Stein, W. H., and Moore, S., *J. Biol. Chem.*, **176**, 337 (1948).
15. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **176**, 367 (1948).
16. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **178**, 53 (1949).
17. Stein, W. H., and Moore, S., *J. Biol. Chem.*, **178**, 79 (1949).
18. Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, **73**, 627 (1927).

AN INVESTIGATION OF THE FOLIC ACID-PROTEIN COMPLEX IN YEAST*

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Specific and reversible combinations between vitamins and proteins have been demonstrated in a number of instances and the coenzyme functions of thiamine, pyridoxal, riboflavin, and nicotinamide are well established on that basis.

Folic acid (FA) exists in yeast largely in the form of a hexaglutamyl conjugate, the glutamic acid residues of which are presumably joined by peptide linkage (1, 2). The modification of the microbiological assay used in the present work (3) offers a satisfactory index of the concentration of the conjugate, but those additional forms of FA which are active only after more involved enzymatic liberation (4) are probably not detected by it.

The recorded data, based chiefly upon microbiological assays with *Lactobacillus casei* after enzymatic hydrolysis, can be interpreted as indicating the association of FA hexaglutamate with the proteins released by autolysis of the yeast cell.

EXPERIMENTAL

Autolysis and Extraction—The procedure for autolysis developed by Meyerhof (5), Kunitz (6), and Cori (7) for the isolation of yeast hexokinase has been modified to permit the extraction of about 5 γ of FA (calculated as the hexaglutamate) per gm. of fresh wet yeast.

Fresh bakers' yeast¹ (*Saccharomyces cerevisiae* Hansen, var. *ellipsoideus*, strain Delft II) was broken into small fragments and suspended in one-half its weight of warm (40°) toluene. The suspension was immersed in a 45° water bath and stirred constantly until a temperature of 40–41° was reached. A transition from a highly viscous gum to a smoothly flowing suspension occurs, and the evolution of CO₂ results in a large increase in volume. During a period of 2.5 hours, the warm suspension was allowed to cool slowly to room temperature. Cold distilled water (about 1 ml.

* This investigation was supported by a grant from the Nutrition Foundation, Inc.

¹ We are indebted to Dr. Alfred S. Schultz of the Research Laboratories of The Fleischmann Company, New York, for large amounts of fresh yeast placed at our disposal.

per gm. of yeast) was then added and the mixture was stored at 0° for 18 hours. The lower aqueous layer, which contains the water-soluble components of the autolyzed yeast, was siphoned off and filtered with suction. (The filter aids recommended by Kunitz for this filtration adsorb FA from aqueous solution and hence were not used. The filtration proceeds rapidly, however, when filter papers (Whatman No. 2) are changed frequently.) All further fractionation was carried out on the clear amber filtrate.

Nature and Stability of FA Protein Complex—The evidence obtained points to the conclusion that the linkage between FA-conjugate and protein is essentially salt-like or, at least, extremely dissociable.

Organic Solvents—Addition of ethanol, acetone, or dioxane to the autolysate or to protein fractions of high FA concentration (prepared by salting-out procedures) results in almost complete cleavage of the FA-protein bond.

Heat—When solutions containing FA conjugate and protein are heated, the protein is precipitated and the conjugate remains in solution, the extent of dissociation depending on the temperature and duration of the heat treatment. Heating for 1 minute at 80°, or for 5 minutes at 60°, for example, released substantially all of the FA hexaglutamate from associated protein.

Dialysis—When freshly prepared autolysates in viscose casing were dialyzed against water, the FA conjugate was rapidly released and passed into the diffusate. In Fig. 1, *A* the results are plotted for dialysis of 100 ml. of autolysate at 20° and pH 6.4 (containing 3.3 γ of FA per ml.) versus 300 ml. of distilled water. At the times indicated by points on the curve, 0.1 ml. aliquots were withdrawn for microbiological assay.

Similar results were obtained for the dialysis of protein fractions of high FA concentration prepared by salting-out procedures (Fig. 1, *B*). The data are plotted for the dialysis of 5 ml. of protein solution (containing 5.4 γ of FA per ml.) at 0° against 15 ml. of distilled water, pH 6.4.

Salt Effects—In the dialysis of "FA-rich" protein fractions against salt solutions, the rate of dissociation depends both on the concentration of the salt and on the pH of the system. In general, the extent of cleavage of the FA-protein bond increases with salt concentration to the salting-out range (at which point the FA conjugate is coprecipitated and remains in the dialysis residue). This effect was observed in solutions of sodium acetate, disodium phosphate, and ammonium sulfate.

In solutions of trisodium citrate, however, an intermediate concentration exists at which the FA-protein complex appears to be more stable than at higher or lower concentrations. In Fig. 2, *A* the results are plotted for the dialysis at 0° of 10 ml. aliquots of a FA-rich protein fraction (about 5 γ of FA per ml.) against 100 ml. aliquots of sodium citrate at pH 7.

The FA content of the residues after 120 hours of dialysis is plotted against the molarity of the diffusate. A maximum occurs at about 0.6 M. A similar experiment, in which the FA concentrations of the dialysis residues

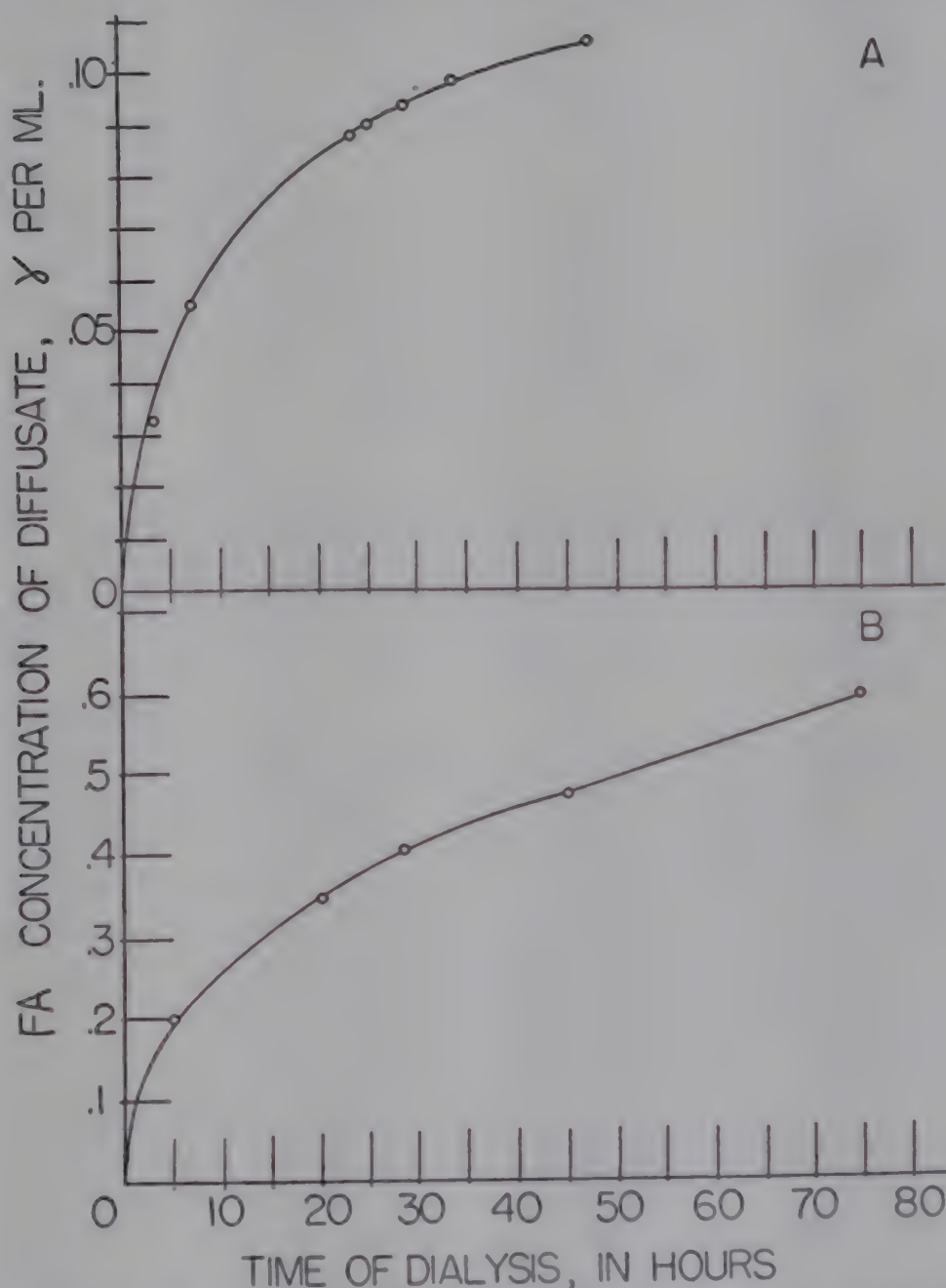


FIG. 1. Rate of dissociation of FA conjugate from associated protein on dialysis against water. The ordinates represent the FA concentration of the diffusate after T hours dialysis of (A) 100 ml. of autolysate at pH 6.4 (containing 3.3 γ of FA per ml.) versus 300 ml. of H_2O , 20°; (B) 5 ml. of an FA-rich protein fraction containing 5.4 γ of FA per ml. versus 15 ml. of H_2O , pH 6.4, 0°.

were obtained by microbiological assay with *Streptococcus faecalis* R, shows a more pronounced maximum than was obtained with *L. casei* assays.

pH Effect—The rate of dissociation of the FA-protein complex depends largely on the pH of the system. At pH values below 4.9 a large amount

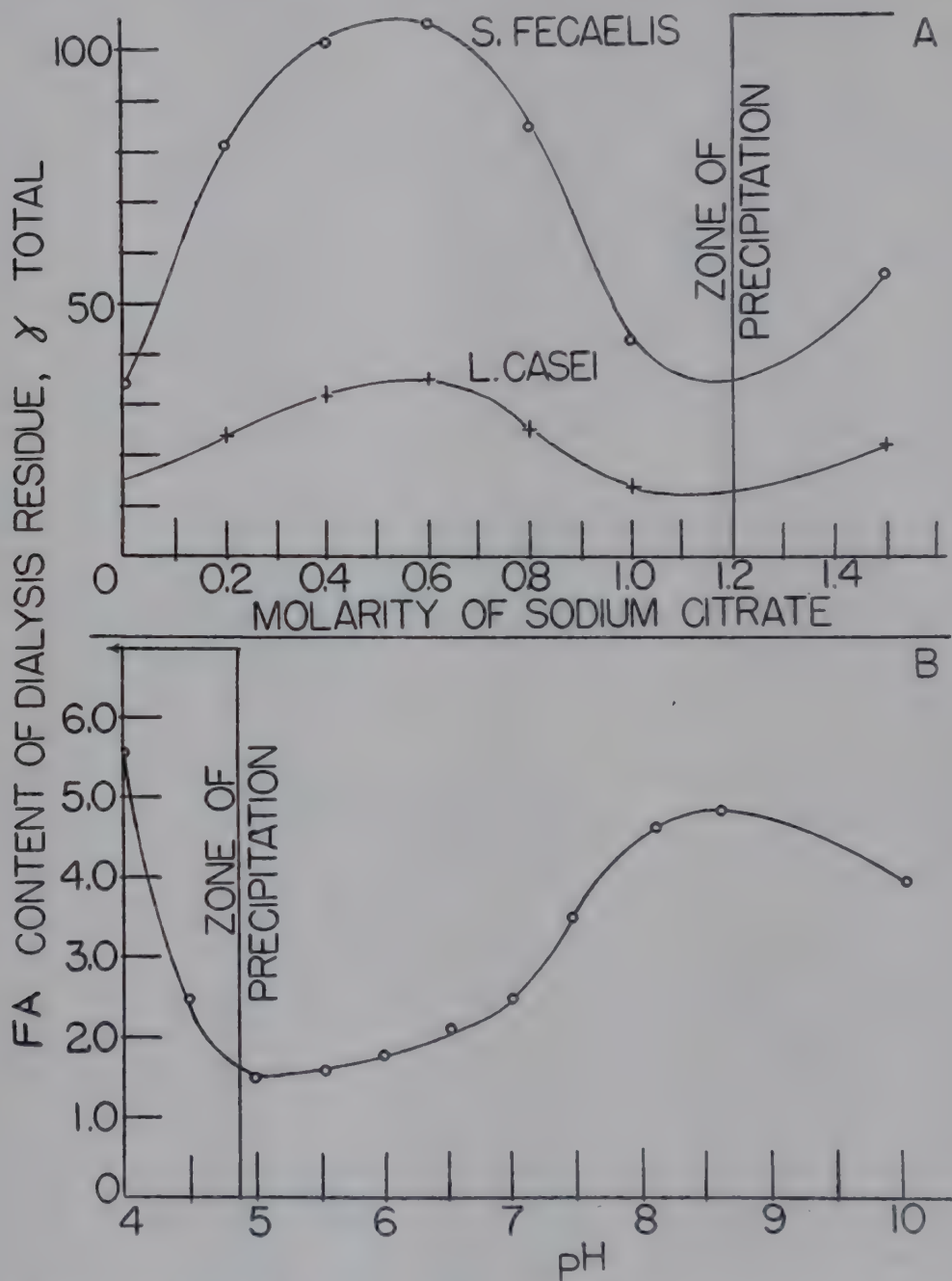


FIG. 2. Stability of the FA-protein complex. A, FA concentration of the dialysis residue as a function of trisodium citrate concentration. The ordinates represent the FA content of 10 ml. aliquots of a FA-rich protein fraction after 120 hours dialysis *versus* 100 ml. of millimolar sodium citrate solution. B, FA concentration of the dialysis residue as a function of pH. The ordinates represent the FA content of 10 ml. of a 1:1 autolysate-buffer mixture after 86 hours dialysis *versus* 95 ml. of 0.2 M buffers at the indicated pH values.

of the FA conjugate is bound to precipitated protein and is non-dialyzable. The pH-stability curve observed in the initial yeast autolysate is plotted

in Fig. 2, *B*. The ordinates on the curve represent the amount of FA remaining in 10 ml. of a 1:1 autolysate-buffer mixture after 86 hours dialysis *versus* 95 ml. of 0.2 M buffers of the indicated pH values. (Acetate buffers were used in the range of pH 4.0 to 5.5, phosphate buffers from pH 6.0 to 7.5, and borate buffers from pH 8.2 to 9.8.) The complex is most stable in the pH range 8.0 to 8.6 and is least stable at pH 5. The approximate linearity of the curve between pH 5 and 7 was verified in similar independent experiments.

Protein Fractionation—Fractionation of yeast autolysates was carried out in aqueous solutions by use of ammonium sulfate in concentrations between 2 and 4 M as the precipitant. At the protein concentrations employed, saturation with sodium sulfate precipitated only about one-third of the amount of FA brought down with $(\text{NH}_4)_2\text{SO}_4$; MgSO_4 did not cause precipitation.

It has been reported that FA conjugates are not precipitable by saturation with ammonium sulfate (8). This was verified in two standard solutions of the FA hexaglutamate (containing 3.4 and 5.3 γ of FA per ml.) by fluorometric analysis (9) before and after saturation with $(\text{NH}_4)_2\text{SO}_4$. The former concentration is approximately that of the yeast autolysate prior to fractionation.

Fractionation Procedure—The stepwise addition of ammonium sulfate to the autolysate produces no precipitation below 2.2 M. At higher concentrations both FA conjugate and protein are precipitated, the extent of precipitation depending on the molarity and pH of the solution. In Fig. 3 the data are plotted for the precipitation of FA conjugate and protein at four pH values. Aliquots of 2 liters of yeast autolysate containing 60 mg. of total solids per ml. (dry weight at 100°) were adjusted to the indicated pH values before and after the addition of solid ammonium sulfate to 2.4 M (360 gm. per liter of autolysate). Solutions were stored at 0° for 17 hours and filtered with suction. Ammonium sulfate concentrations (expressed as moles per liter on the abscissae of Fig. 3) were calculated from the volumes of the filtrates and the total amounts of $(\text{NH}_4)_2\text{SO}_4$ added to each.² The precipitates were redissolved in water (approximately 2 ml. per gm. of precipitate). Protein concentrations were determined as the difference between total solids and the concentrations of ammonium sulfate as determined colorimetrically with Nessler's reagent (10). FA concentrations were determined microbiologically with *L. casei*.

The filtrates obtained at 2.4 M were brought to 2.6 M with ammonium sulfate (54 gm. per liter) and adjusted to the required pH. After storage

² Molarities calculated in this way represent the concentration which would exist if successive protein precipitates were not removed. They do not include a correction for $(\text{NH}_4)_2\text{SO}_4$ bound to precipitated protein.

for 17 hours at 0°, the solutions were filtered with suction. The FA and protein concentrations of the redissolved precipitates were determined as before.

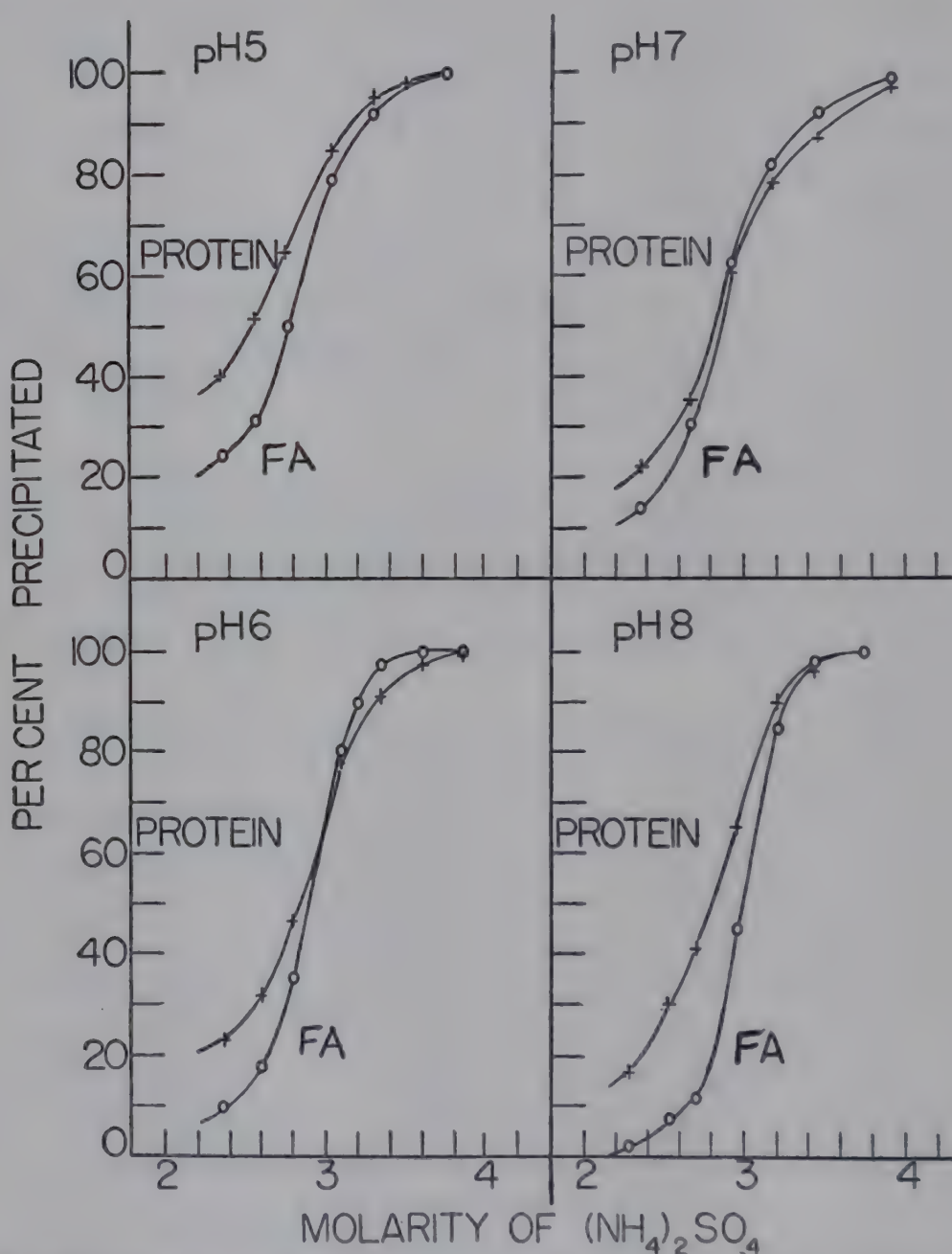


FIG. 3. Precipitation curves of protein and FA conjugate. The ordinates represent the per cent of the total precipitable protein and FA conjugate salted-out by millimolar (NH₄)₂SO₄ at the indicated pH values.

The stepwise addition of ammonium sulfate to the filtrates was continued in this fashion to 3.8 M, beyond which point no further precipitation occurred. The amounts of FA conjugate and protein brought down in successive precipitates are expressed as per cent of the *total* quantities precipitable over the entire salting-out range. The additive percentages are then plotted against the corresponding concentrations of (NH₄)₂SO₄.

A number of conclusions can be drawn from inspection of Fig. 3 and related salting-out data.

The slope of the linear portion of the FA precipitation curve is greater than that of the corresponding protein precipitation curve at all pH values examined. Since the FA conjugate is not itself precipitable under these conditions, it follows that it is specifically bound to one or more of the proteins precipitated by high concentrations of $(\text{NH}_4)_2\text{SO}_4$. If the conjugate were non-specifically adsorbed on all proteins in the autolysate, the FA and protein precipitation curves would be superimposed roughly over the entire concentration range.

The total quantity of protein precipitated between 0 and 3.8 M varies but slightly with the pH of the series. The over-all yields obtained at pH 5, 6, and 7 are approximately equal (9 gm. of protein per liter of autolysate). The yields at pH 8 are about 6 per cent lower.

The total amount of the FA conjugate coprecipitated, however, depends largely on the pH of the system. At pH 5, for example, the over-all yields average 180 γ of FA per liter of autolysate, in comparison with 470 γ per liter at pH 6, and 590 γ per liter at pH 8. Only about 20 per cent of the total FA content of the autolysate is precipitable in the pH range 6 to 8.

Evidence of a specific association between FA conjugate and protein is obtained by calculating the amount of FA bound per gm. of protein in the successive fractions obtained by the salting-out procedure. Histograms representing the FA concentrations of the various protein precipitates are shown in Fig. 4. Micrograms of FA per gm. of protein are plotted as a constant ordinate over the range of $(\text{NH}_4)_2\text{SO}_4$ concentration within which each fraction was collected.

Inspection of the histograms reveals that salt fractionation at pH 5 does not lead to the separation of an FA-rich protein precipitate. This result is in accord with the evidence of dialysis experiments which show that the FA-protein complex is least stable at this pH.

Salt fractionation at pH 6 yields three active precipitates in the concentration range 2.7 to 3.4 M, with a maximum of 80 γ of FA per gm. of protein precipitated between 2.8 and 3.1 M. The data obtained at pH 7 do not differ appreciably from these results.

At pH 8 a maximum of 120 γ of FA per gm. of protein precipitated is observed between 2.95 and 3.2 M. This is considerably higher than the maxima obtained at lower pH values and verifies the observation in dialysis experiments that the FA-protein complex is more stable in mildly alkaline solution.

A 7-fold difference in FA content between proteins precipitated at 2.4 M and those precipitated between 2.95 and 3.2 M is indicative of the existence of a specific FA conjugate-protein complex in the yeast autolysate.

Electrophoretic Analysis—In an attempt to correlate FA concentration

with protein composition, the various fractions obtained by the stepwise addition of ammonium sulfate at pH 6 were examined in the Tiselius apparatus at pH 7.1 and 7.7 (Table I).

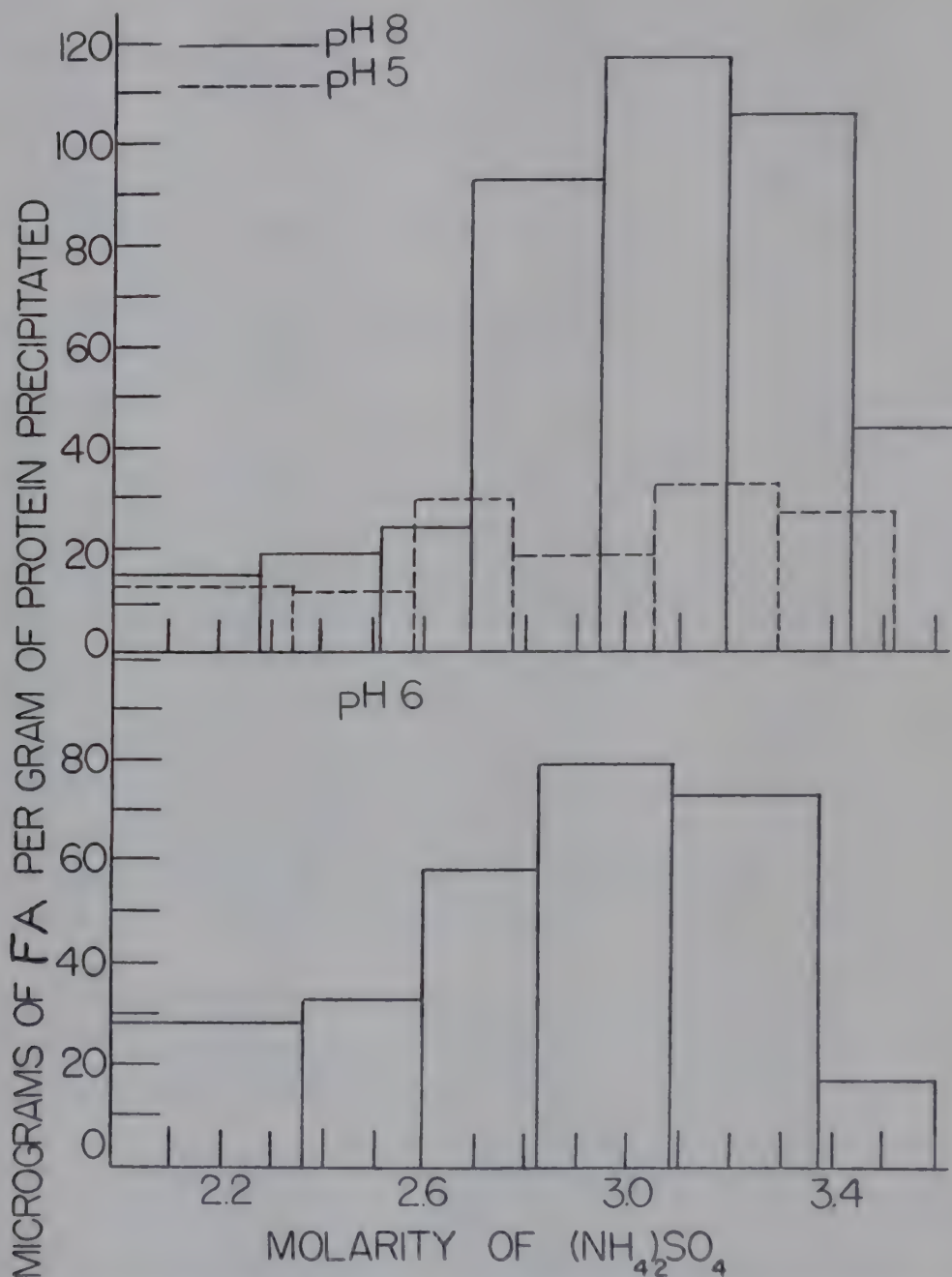


FIG. 4. Activity ratios of the precipitates obtained by ammonium sulfate fractionation. The number of micrograms of FA per gm. of protein is plotted as a constant ordinate over the range of salt concentration at which the fraction is obtained.

The initial autolysate and the protein precipitates obtained by salt fractionation can be interpreted in terms of seven components in the electrophoretic pattern (Fig. 5). The FA concentrations of the separate fractions and their protein compositions as determined by planimeter

measurement of the peak areas³ are given in Table I. The data represent the average of the relative areas of both ascending and descending boundaries. Peak 6 represents, at least in part, an immobile salt boundary

TABLE I
Electrophoretic Analysis of Yeast Protein Preparations in Phosphate Buffers

Frac- tion No.	Salting-out range	Activity ratio*	Per cent composition						
			Compo- nent 1	Compo- nent 2	Compo- nent 3	Compo- nent 4	Compo- nent 5	Compo- nent 6	Compo- nent 7
Ionic strength 0.2, pH 7.1									
I	0 -2.41	22	3.9	2.3	16.5	49.8	11.4	12.7	3.6
			(4.5)	(2.7)	(19.0)	(57.0)	(13.0)		(4.1)
II	2.41-2.71	42	3.2	1.5	11.8	65.5	10.9	4.5	2.8
			(3.4)	(1.6)	(12.3)	(68.5)	(11.3)		(2.9)
III	2.71-2.96	99	2.8	0.8	12.5	64.0	13.5	4.0	2.8
			(2.9)	(0.9)	(13.0)	(66.6)	(13.8)		(2.9)
IV	2.96-3.23	72	4.0	1.4	19.1	43.2	21.0	8.7	2.8
			(4.3)	(1.5)	(21.0)	(47.4)	(23.0)		(3.0)
V	3.23-3.48	37	4.3	1.1	21.3	32.2	30.2	9.0	2.1
			(4.8)	(1.3)	(23.4)	(35.3)	(33.1)		(2.1)
VI	3.48-3.84	28	0	3.6	12.5	15.0	64.7	4.5	0
			(0)	(3.7)	(13.0)	(15.8)	(67.7)		(0)
Ionic strength 0.2, pH 7.7									
I	0 -2.34	28	5.9	2.6	17.5	56.1	9.4	8.9	2.6
			(6.4)	(2.8)	(19.3)	(61.7)	(10.3)		(2.8)
II	2.34-2.58	33	7.5	7.4	13.4	46.0	16.3	4.8	4.8
			(7.9)	(7.7)	(14.4)	(48.3)	(17.1)		(5.0)
III	2.58-2.74	59	5.2	2.1	15.6	51.7	9.5	7.2	8.9
			(5.5)	(2.3)	(16.8)	(55.6)	(10.2)		(9.7)
IV	2.74-3.00	72	4.9	1.6	10.8	57.9	8.4	11.2	5.2
			(5.4)	(1.8)	(12.3)	(65.3)	(9.4)		(5.8)
V	3.00-3.20	67	7.8	0.4	21.9	34.5	17.0	13.9	4.8
			(9.1)	(0.5)	(25.4)	(40.0)	(19.5)		(5.5)
VI	3.20-3.45	26	7.1	2.5	25.3	29.5	23.6	8.3	3.7
			(7.7)	(2.6)	(27.7)	(32.2)	(25.7)		(4.0)

* Activity ratio expressed as micrograms of FA per gm. of protein.

formed as a result of differences in buffer ion concentration between the protein and buffer solutions. When the area of the peak is subtracted

³ Planimeter measurements were made on an enlarged projection of the pattern. Subdivision into peaks was achieved by dropping perpendiculars from minima in the curve to the horizontal base-line.

from the total area of the pattern in the computation of protein composition, one obtains the corrected values given in parentheses in Table I.

The data indicate a possible association of FA activity with one of the proteins in peak 4. However, because of the complexity of the mixtures

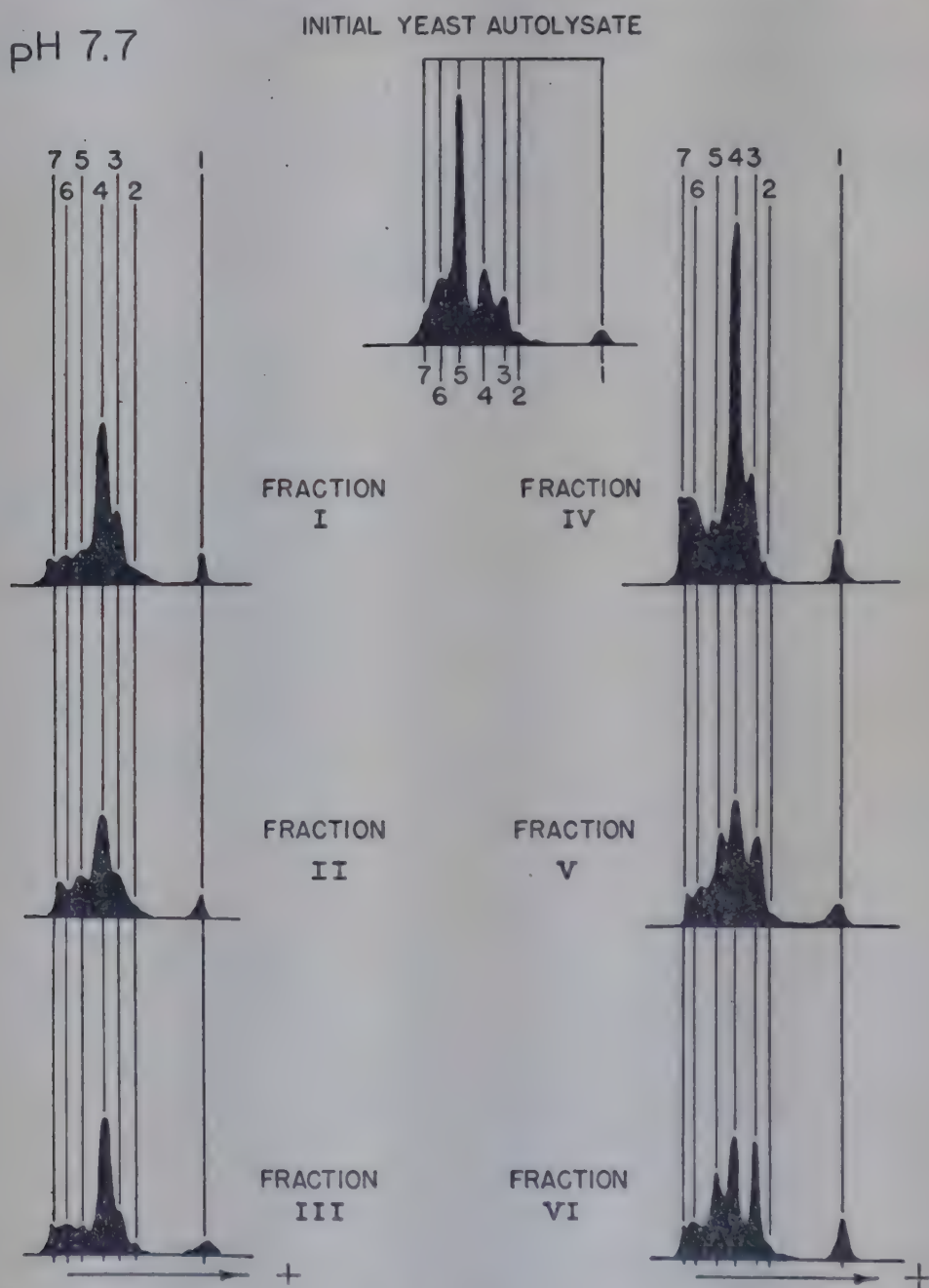


FIG. 5. Electrophoretic patterns of the precipitates obtained by ammonium sulfate fractionation of yeast autolysates. Patterns obtained in phosphate buffers of ionic strength 0.2 and pH 7.7.

obtained by a single ammonium sulfate fractionation, a direct correlation between FA concentration and that of a particular electrophoretic component is not observed.

Crystallization Procedures—Two major approaches to the isolation of the FA-protein complex were attempted. Both involved fractional crystalliza-

tion of proteins from solutions of the precipitates obtained by ammonium sulfate fractionation.

Method I—All of the FA activity precipitable at pH 6 between 2.4 and 3.1 M was collected in a single fraction. This step rejected half of the protein but only 30 per cent of the FA. Fractional crystallization of the mixture yielded three distinct crystalline proteins, none of which, however, contained significant amounts of FA conjugate.

The detailed procedure was as follows: The initial autolysate (60 mg. of total solids per ml.) was brought to 2.4 M by the addition of 350 gm. of $(\text{NH}_4)_2\text{SO}_4$ per liter. After storage for 17 hours at 0° , the solution was filtered and the precipitate was discarded. The clear filtrate was brought to 3.1 M with ammonium sulfate (106 gm. per liter of autolysate), stored at 0° for 17 hours, and then filtered with suction. The filtrate was discarded.

The precipitate was redissolved in water (1 ml. per gm. of precipitate), and solid ammonium sulfate was added until the solution showed just a trace of turbidity.⁴ The solution was then titrated to pH 7.2 to 7.3 with 1 N NaOH and stored at $0-5^\circ$ for 10 to 14 days. An interlocking mass of needle-shaped crystals was formed in this period (Fig. 6, A) and was removed by filtration. The crystals contained no FA and no riboflavin. They have been tested for tyrosinase, dopa decarboxylase, amylase, and protease activity and found to be inactive. After a single recrystallization, they were found to be electrophoretically homogeneous (Fig. 7, A). (The first crystals prepared generally contained a significant amount of amorphous material which appears in the electrophoretic pattern (Fig. 7, B).) When the clear filtrate was readjusted to turbidity under the same conditions, more of the crystals generally appeared within 48 hours, although, in some cases, clusters of crystalline tyrosine were observed.

The procedure was modified, also, to permit the isolation of a second crystalline protein. The filtrate was diluted to twice its volume with cold distilled water, and solid ammonium sulfate was added to a trace of turbidity. (The protein concentration at this point is about 60 mg. per ml.) The solution was then titrated to pH 7.5 and stored at 0° for 10 days. The crystals (Fig. 6, B), collected by centrifugation, were electrophoretically homogeneous at pH 7.2 and 7.7 (Fig. 7, C, D). They did not contain significant amounts of FA. Both microbiological and chemical analyses (9) showed less than 100 γ of FA per gm. of protein. Solutions of the crystals were tested for amylase, protease, tyrosinase, and dopa decarboxylase activity and were found to be inactive.

Further fractionation of the filtrate was complicated by proteolysis, as

⁴ The results obtained depend to a great extent on the protein concentration. The indicated pattern of crystallization was obtained in solutions containing about 140 mg. of protein per ml.

indicated by continuous deposition of crystals of tyrosine. When the filtrate was readjusted to turbidity at pH 7.5, a brown⁵ precipitate of apparently crystalline material was formed after 4 to 6 weeks at 0°. This material was not electrophoretically homogeneous (Fig. 7, *E*). In a few cases the crystals contained FA in significant concentrations (about 140 γ per gm. of protein), but in those instances they were associated with amor-

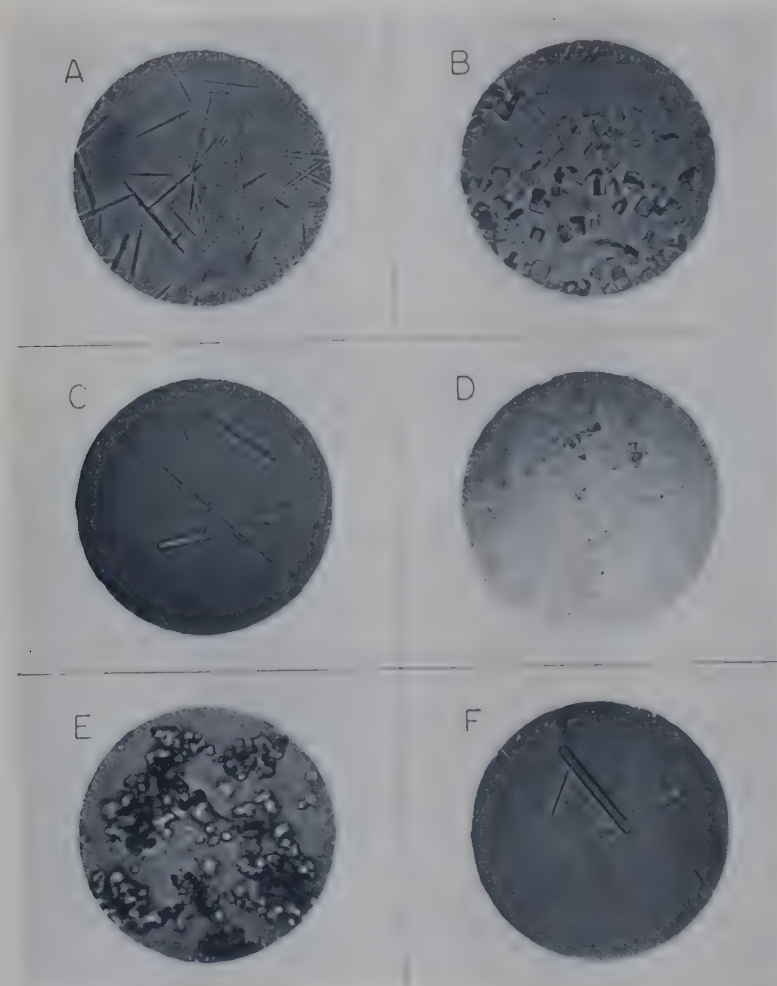


FIG. 6. Photomicrographs of crystalline proteins obtained from yeast autolysates by salting-out procedures. *A*, protein crystals precipitable between 2.60 and 2.77 M $(NH_4)_2SO_4$; *B*, protein crystals precipitable between 2.77 and 3.05 M $(NH_4)_2SO_4$; *C*, crystalline hexokinase prepared by Method I; *D*, protein crystals prepared by sodium citrate-ammonium sulfate precipitation; *E*, crystals of brown protein precipitable between 0 and 2.34 M $(NH_4)_2SO_4$; and *F*, crystalline hexokinase prepared by Method II.

phous material. Mild protease activity was observed on incubation with suspensions of denatured ovalbumin. Recrystallization furnished excellent preparations of Crystals B.

Further crystallization from the filtrate yielded crystalline hexokinase

⁵ The dark pigment did not dissociate on dialysis. In the Tiselius cell however, it did not migrate with the major electrophoretic component.

(Fig. 6, C). The enzyme was prepared more simply, however, by Method II.

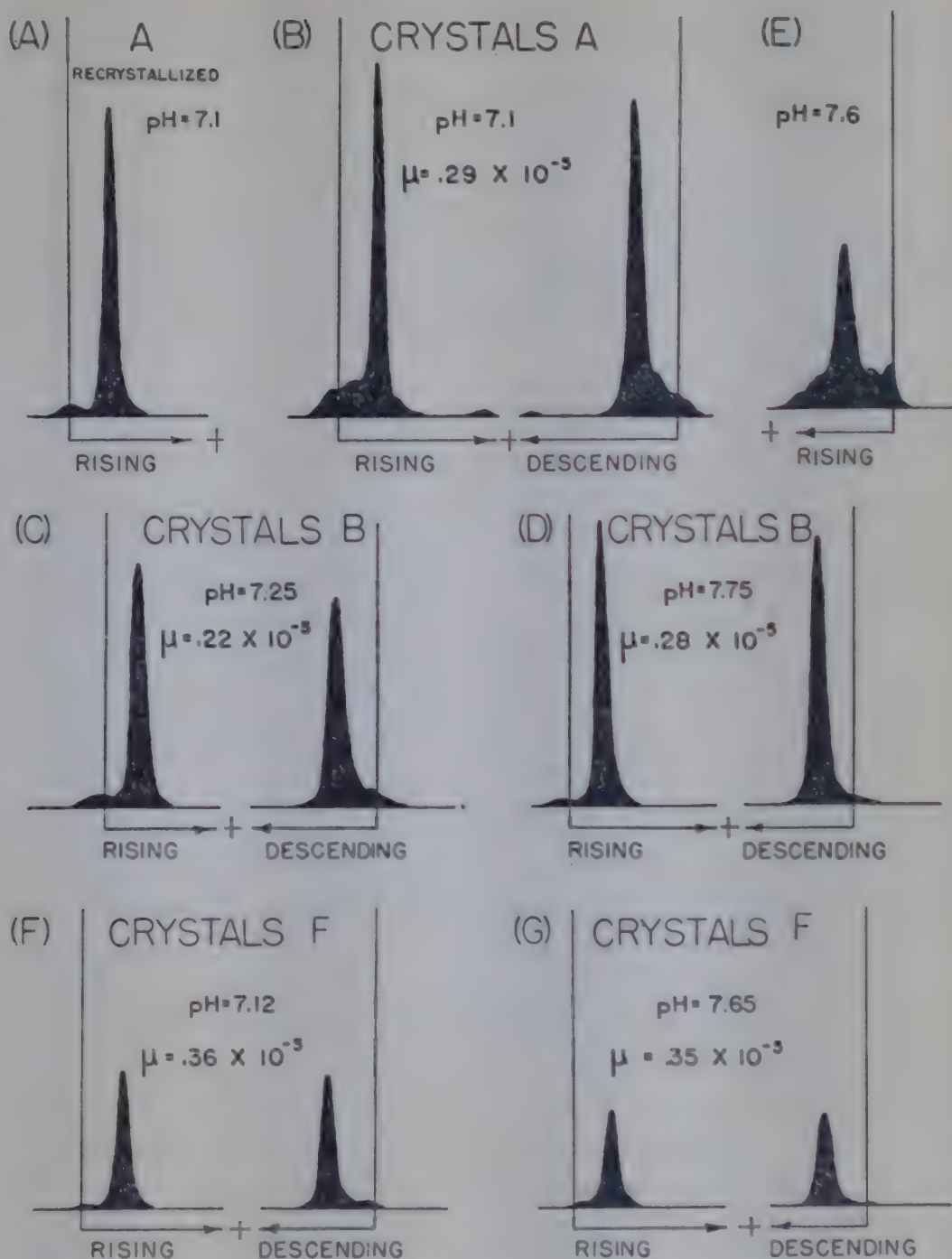


FIG. 7. Electrophoretic patterns of crystalline protein preparations. Mobilities (μ) given in sq. cm. per volt per second. (A) recrystallized preparation of protein precipitable between 2.60 and 2.77 M $(\text{NH}_4)_2\text{SO}_4$, (B) protein (A) prior to recrystallization, (C) and (D) protein crystals precipitable between 2.77 and 3.05 M $(\text{NH}_4)_2\text{SO}_4$, (E) brown protein precipitable between 2.4 and 3.1 M $(\text{NH}_4)_2\text{SO}_4$, and (F) crystalline hexokinase.

Effects of Citrate—Although the FA-protein complex can be shown to be stable in solutions of trisodium citrate, this salt cannot be incorporated

with facility into the isolation procedure. In high concentrations, it reduces the autolysis yield considerably, and saturation with sodium citrate does not precipitate an FA-rich protein fraction. Ammonium sulfate fractionation of solutions already 0.6 M in sodium citrate leads to the precipitation of Na_2SO_4 before the FA-active fractions have been precipitated. One such adaptation, however, permits the isolation of a fourth crystalline protein. The autolysate is brought to 0.6 M sodium citrate, and ammonium sulfate is then added to 2.4 M. The precipitate formed is redissolved in an equal weight of water and the solution is brought to 1.9 M with $(\text{NH}_4)_2\text{SO}_4$. The redissolved precipitate is adjusted to turbidity at pH 7.6 and stored for 2 to 3 weeks at 0° . The crystals formed (Fig. 6, *D*) do not contain significant amounts of FA.

Method II—The stepwise addition of ammonium sulfate to the autolysate produces a series of protein mixtures which can be further resolved by means of fractional crystallization. The fractionation of the yeast autolysate into protein mixtures of varying FA concentration has been described. The resolution of such mixtures by fractional crystallization or by repetition of the salting-out procedure was next accomplished.

All of the precipitates resulting from the stepwise addition of ammonium sulfate to the autolysate were treated in approximately the same way. The fractions were redissolved in water (2 ml. per gm. of precipitate), filtered, and adjusted to turbidity by the addition of solid $(\text{NH}_4)_2\text{SO}_4$. Further details of manipulation are described in the subsequent summary of the crystallization phenomena observed for the individual fractions.

Fraction I—The salting-out range is 0 to 2.34 M $(\text{NH}_4)_2\text{SO}_4$ at pH 6. This first fraction contained about 20 per cent of the protein and about 10 per cent of the precipitable FA activity of the initial autolysate. Its activity ratio (micrograms of FA per gm. of protein) was 23.⁶

After preliminary adjustment to turbidity, the solution (containing 75 to 80 mg. of protein per ml.) was titrated to pH 7.2 and stored at 0° for 2 to 3 weeks. A mixed crystalline precipitate of tyrosine and protein was formed. The precipitate was suspended in an equal weight of water and filtered clear of the comparatively insoluble crystals of tyrosine. The filtrate was then brought to turbidity at pH 7.2 and stored at 0° for 1 to 2 weeks.

The clusters of almost spherical crystals (Fig. 6, *E*) were collected by centrifugation. The crystals contained less than 30 γ of FA per gm. They were associated with a dark pigment which did not dissociate on dialysis. Solutions of the crystals gave negative tests for activity of amylase, tyrosinase, dopa decarboxylase, and xanthopterin oxidase. They showed slight proteolytic activity.

⁶ The activity ratios presented are the average values of eleven fractionations. All values agreed to within ± 10 per cent of the mean.

Fraction II—The salting-out range is 2.34 to 2.6 M $(\text{NH}_4)_2\text{SO}_4$ at pH 6. Fraction II contained 10 per cent of the protein and 6 per cent of the precipitable FA activity. Its activity was equivalent to 33.

When the turbid solution (containing 70 mg. of protein per ml.) was titrated to pH 7.5 and stored at 0° for 1 to 2 weeks, small rhombic plates of a yellow protein were deposited. These crystals were identical in form with the crystals of "yeast yellow protein" isolated by Kunitz (6). The crystals contained less than 30 γ of FA per gm.

The pattern of crystallization observed in Fraction II often resembled that of Fraction I in the deposition of tyrosine and a dark crystalline protein. Because of the low activity of both fractions, the procedures were not further investigated.

Fraction III—The salting-out range is 2.60 to 2.77 M. Fraction III contained 15 per cent of the protein and 17 per cent of the precipitable FA activity. When adjusted to turbidity at pH 7.2 and stored at 0° for 1 to 2 weeks, the solution (containing 70 to 80 mg. of protein per ml.) deposited a fine network of needle-like crystals. The crystals were identical in appearance and in electrophoretic mobility with the Crystals A prepared by Method I. They did not contain FA.

Fraction IV—The salting-out range is 2.77 to 3.05 M. Fraction IV contained 32 per cent of the protein and 45 per cent of the precipitable FA activity. Its activity was 80 γ of FA per gm. of protein. When adjusted to turbidity at pH 7.5 and stored at 0° for 1 to 2 weeks, the solution (containing about 100 mg. of protein per ml.) produced a mass of small crystals, identical in appearance and in electrophoretic mobility with the Crystals B prepared by Method I. The crystals contained less than 30 γ of FA per gm.

Refractionation—Fraction IV was refractionated by the stepwise addition of ammonium sulfate, with results which are indicated graphically in Fig. 8. The procedure was as follows: Fraction IV⁷ was redissolved to give a solution containing 30 mg. of protein per ml. The pH was adjusted to 8 before and after the addition of successive portions of ammonium sulfate. The precipitates formed were redissolved in 2 volumes of water and adjusted to turbidity at pH 8.2. Protein and FA concentrations were determined as before, and the activity of each subfraction (micrograms of FA per gm. of protein) was plotted as a constant ordinate against the concentration range at which the subfraction was obtained.

As indicated in Fig. 8, a maximum activity ratio of 160 was obtained in the concentration range 2.8 to 3.2 M. Crystallization of subfractions gave crystals similar in appearance and in electrophoretic mobility to

⁷ Obtained by salt fractionation at pH 6. Similar refractionation of FA-active precipitates salted-out at pH 8 does not result in a significant concentration of FA activity.

Crystals B of Fig. 6. This material contained less than 50 γ of FA per gm. No other crystal forms were observed in these high activity fractions. Recrystallization of Subfraction D resulted in relatively pure, crystalline hexokinase⁸ (Fig. 6, *F*).

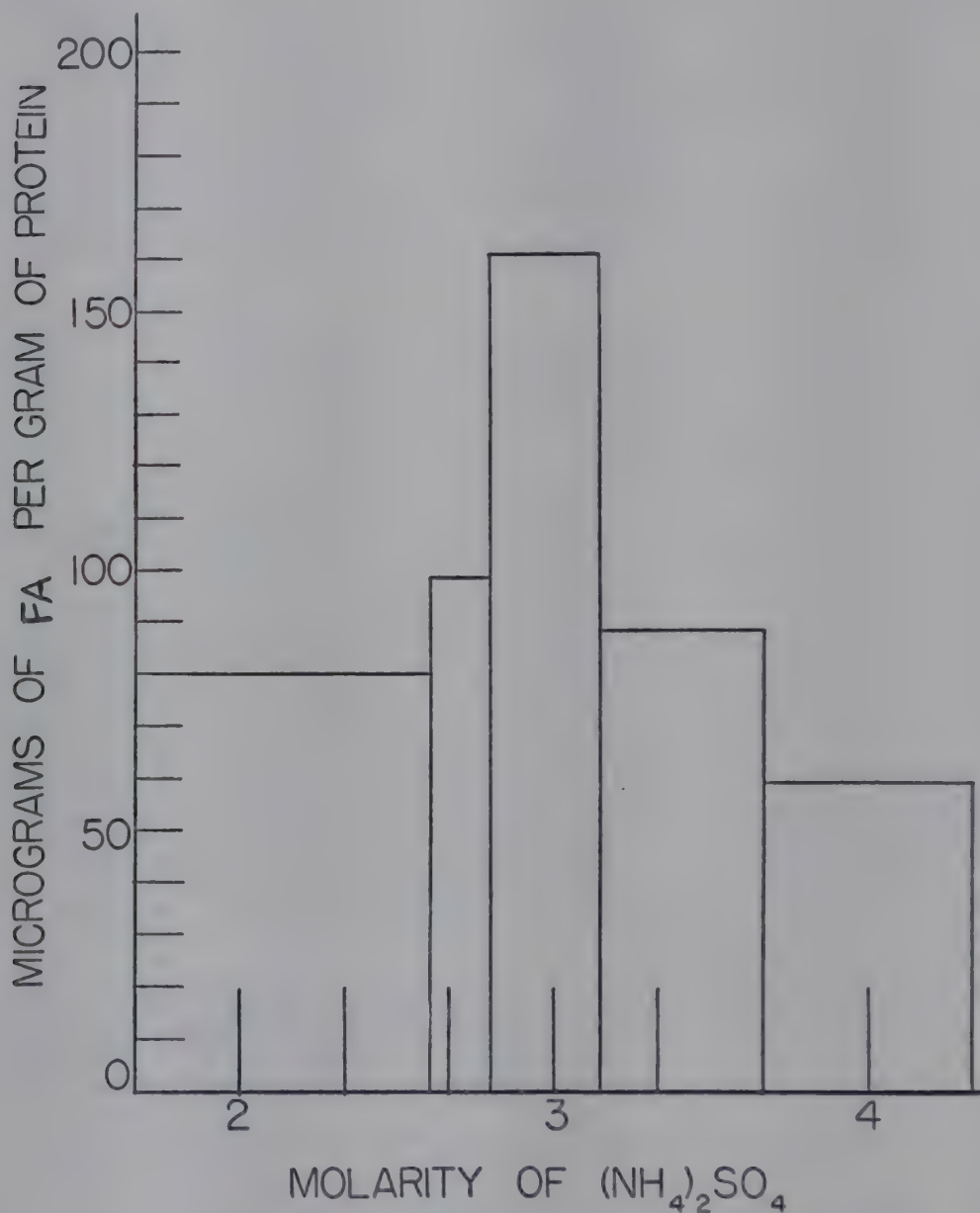


FIG. 8. Activity ratios of the precipitates obtained by refractionation of Fraction IV. The number of micrograms of FA per gm. of protein is plotted as a constant ordinate over the range of salt concentration at which the subfraction is obtained.

Fraction V—The salting-out range is 3.05 to 3.22 m. Fraction V contained about 14 per cent of the protein and about 18 per cent of the precipitable FA activity. Its activity was 70. Adjustment to turbidity

⁸ The authors are indebted to Dr. L. J. Teply of the Institute for Enzyme Research, University of Wisconsin, for making hexokinase activity tests and for other assistance during initial stages of the investigation.

at pH 7.5 resulted in the deposition of small, spherical crystals which contained 60 to 75 γ of FA per gm. of protein.

Fraction VI—The salting-out range is 3.22 to 3.43 M. Fraction VI contained about 8 per cent of the protein and about 3 per cent of the FA activity. Adjustment to turbidity at pH 7.5 and storage at 0° for 2 to 3 weeks resulted in the deposition of long crystals of hexokinase (Fig. 6, *F*). The crystals were electrophoretically homogeneous at pH 7.1 and 7.65 (Fig. 7, *F*, *G*). They did not contain FA.

DISCUSSION

The supposition that FA resembles other members of the vitamin B complex in the formation of a specific combination with protein is sustained by examination of its distribution in precipitates obtained by ammonium sulfate fractionation. The existence of protein fractions high in FA concentration is of interest in relation to the foregoing suggestion, but it does not constitute proof of a specific functional association. In the analogous cases of the flavo- and pyridinoproteins, conclusive proof of a specific vitamin-protein bond was based chiefly upon demonstration of an enzyme function which neither component alone could fulfil.

The difficulties and uncertainties in microbiological assays for FA are inherent in the FA to protein ratios reported. Even under closely controlled conditions these variations may amount to 10 per cent of the concentration observed. It is significant that the FA in all the precipitates analyzed requires enzymatic liberation. Since the test organism (*Lactobacillus casei*) can utilize the triglutamyl conjugate of folic acid, it is likely that the coprecipitated FA occurs almost entirely as the hexaglutamate.

The recorded crystallization procedures have been checked in at least four instances in each case, and they are believed to be readily reproducible. Three of the crystal forms isolated (Fig. 6, *A*, "yeast yellow protein," and crystalline hexokinase) have been prepared previously by Kunitz (6).

A full discussion of electrophoretic analysis by the moving boundary method, as used, will be found in the extensive theoretical and experimental study by Svensson (11).

SUMMARY

The results of ammonium sulfate fractionation of yeast autolysates, supplemented by electrophoretic studies, indicate the presence of a specific FA-protein complex. A method is presented for the preparation of protein fractions containing about 160 γ of folic acid (as the hexaglutamate) per gm. of protein.

Procedures are outlined, also, for the isolation of six crystalline yeast proteins, one of which has been shown to be crystalline hexokinase.

BIBLIOGRAPHY

1. Pfiffner, J. J., *Science*, **102**, 222 (1945).
2. Pfiffner, J. J., Calkins, D. G., Bloom, E. S., and O'Dell, B. L., *J. Am. Chem. Soc.*, **68**, 1392 (1946).
3. Olson, O. E., Fager, E. E. C., Burris, R. H., and Elvehjem, C. A., *Arch. Biochem.*, **18**, 261 (1948).
4. Sreenivasan, A., Harper, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, **177**, 117 (1949).
5. Meyerhof, O., *Biochem. Z.*, **183**, 176 (1927).
6. Kunitz, M., and McDonald, M. R., *J. Gen. Physiol.*, **29**, 393 (1946).
7. Berger, L., Slein, M. W., Colowick, S. P., and Cori, C. F., *J. Gen. Physiol.*, **29**, 379 (1946).
8. Wills, L., Clutterbuck, P. W., and Evans, P. D. F., *Biochem. J.*, **31**, 2136 (1937).
9. Allfrey, V., Teply, L. J., Geffen, C., and King, C. G., *J. Biol. Chem.*, **178**, 465 (1949).
10. Koch, F. C., and McMeekin, T. L., *J. Am. Chem. Soc.*, **46**, 2066 (1924).
11. Svensson, H., *Ark. Kemi*, **22**, No. 10 (1946).

STUDIES ON THE PURITY AND SPECIFICITY OF CYTOCHROME *c*

I. ELECTROPHORETIC ANALYSES

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In a program for the production of cytochrome *c* for therapeutic trials, it was important to consider criteria for testing the purity of the final product. Some reports on the isolation of this material from various animal sources considered an iron content of 0.34 per cent to represent ultimate purity (1-3). Later, Theorell and Åkesson (4, 5) observed cytochrome *c* of this degree of purity to be separable in the Tiselius electrophoresis apparatus into two components. After removal of a smaller, colorless component, the purified cytochrome migrated as a homogeneous substance in the electric field and had an iron content of 0.43 per cent. Subsequently, Keilin and Hartree (6) described a salt fractionation procedure which yielded cytochrome *c* of this higher iron content. However, they believed that the colorless protein so removed represented a part of the intracellular cytochrome *c* rather than an indifferent impurity associated during the extraction procedure.

In view of this lack of agreement on the absolute iron content of pure cytochrome *c*, the criterion of purity adopted as standard in this program was based upon the electrophoretic method and was thus independent of iron analysis. However, in preliminary electrophoretic observations on cytochrome *c* preparations from mixed animal sources, more than a single major component was occasionally observed migrating within the mobility range reported for cytochrome *c* (5). This phenomenon suggested some degree of species specificity of cytochrome *c*, at least with respect to electrophoretic properties. Accordingly, an investigation was undertaken to examine the electrophoretic properties of cytochrome *c* products derived from several pure generic sources: horse, beef, pig, and chicken. The results of some of these studies are reported in this paper.

Preparation

Animal hearts as source material for cytochrome *c* extractions were collected immediately after slaughter, perfused with cold tap water and drained, and refrigerated until adequate starting quantities were accumulated. The organs of the larger species (horse and beef) were manually

trimmed of fat and connective tissue; those of the smaller varieties (pig and chicken) were processed without trimming. The various batches were individually ground by an electrically driven meat chopper through a $\frac{1}{8}$ inch perforated plate. Each lot was then processed in the cold with tap water according to the method of Keilin and Hartree (3, 6). The starting weights of ground meat for the several runs were the following: beef, 136 kilos; horse, 136 kilos; pig, 125.6 kilos; and chicken, 22.7 kilos.

The general extraction procedure was modified to the extent that the precipitation stages with $(\text{NH}_4)_2\text{SO}_4$ and trichloroacetic acid were performed twice, the second time in distilled water. The final precipitates were dissolved in water to a concentration of 12 to 18 mg. per ml. (solids basis), adjusted to neutral pH, and then were dialyzed in cellophane sacs against repeated changes of 0.5 per cent NaCl in the cold until sulfate-free. Varying small amounts of precipitate, generally pale yellow to gray in color, were discarded.

The approximate concentration of the pigment in each of these preparations was determined gravimetrically with correction for NaCl. The final weights of the products thus calculated were as follows: beef, 8.04 gm.; horse, 11.65 gm.; pig, 15.30 gm.; and chicken, 1.13 gm. The exact purities of these preparations were then determined by electrophoretic analyses of the saline preparations as subsequently described.

Electrophoresis

The electrophoretic investigations were carried out at 0.5° in the apparatus described by Longworth (7, 8). To record the refractive index gradients in the optically dense cytochrome solutions, in concentrations ranging from 10 to 15 mg. per ml., a tungsten filament light source was adequate. However, the intensity of illumination required to activate fully even very rapid panchromatic film was generally excessive for the colorless portions of the cell solution. With the consequent overexposure of the film in the latter areas, the sharpness of the patterns was lost by halation and was accompanied by a perceptible shift of the base-line. Some control of this situation was possible by a combination of two methods. A suitable red filter (Wratten, A, orange-red) in the path of the illumination to the cell tended to equalize the light transmitted by the colored and colorless portions of the cell. Furthermore, the optical density of the colored portions was decreased by using reduced cytochrome *c* solutions, allowing less intense illumination. Preliminary studies demonstrated no discernible alterations in the electrophoretic properties of cytochrome *c* preparations as influenced by the state of oxidation or reduction of the prosthetic group. Consequently, the procedure adopted for preparing all samples was to make up the solution of the pigment in a suit-

able buffer, add twice the calculated amount of sodium dithionite to effect complete reduction, and then dialyze to ionic equilibrium against excess buffer (100 volumes or more). Although Keilin and Hartree (3) have indicated that cytochrome *c* is not autoxidizable between pH 4 and 12, the reduced preparations in buffers covering this range showed some degree of autoxidation as excess reducing agent was lost in dialysis. This tendency was more pronounced in the more acid buffers. The typical absorption spectrum of fully reduced cytochrome *c* could be restored by addition of more reducing agent. However, the preparations were used without further treatment following dialysis.

In preliminary electrophoresis in buffers of 0.1 ionic strength, or less, the patterns obtained were markedly asymmetrical. At moderately low field strengths and brief exposure times, high sensitivity to electrode effects was frequently evident from the disintegration of the patterns,

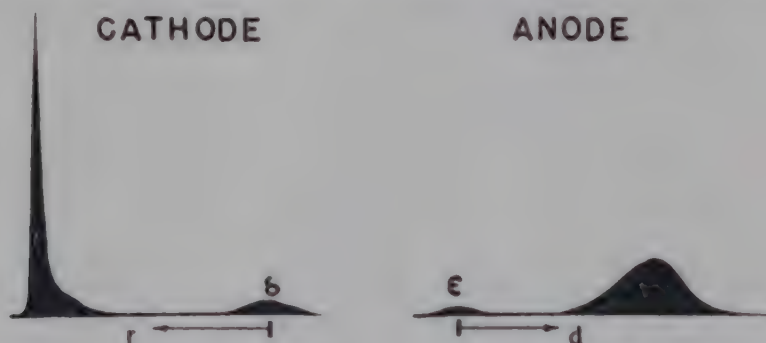


FIG. 1. Electrophoretic diagrams of horse cytochrome *c*; protein concentration, 1 per cent. Phosphate buffer, pH 5.45; 0.10 ionic strength; duration of run, 186 minutes at 6.12 volts per cm.; ϵ and δ , stationary buffer boundaries on rising (*r*) and descending (*d*) sides.

generally into spiked convection boundaries, and, particularly on the descending side, the migrating protein usually formed an extended zone with indefinite boundary surfaces (Fig. 1). Similar observations were recorded by Theorell and Åkesson (5) in electrophoretic studies with buffers of the same ionic strength; however, they concluded that the multiplicity of the boundaries on the descending side arose from the formation of dissociable salt complexes between cytochrome *c* and the impurities present in the preparation. In the present studies, however, this phenomenon was evident in these buffers even in a preparation (horse) which subsequently demonstrated no detectable impurity. Normal electrophoretic patterns of cytochrome *c* were readily obtained by increasing the buffer ionic strength to levels of 0.15 to 0.20. Under these conditions, the patterns were nearly mirror images, and with extended running time to secure adequate migration and separation of cytochrome *c* from its impurities, the formation of false boundaries was almost completely

TABLE I
Electrophoretic Fractionation of Cytochrome c Preparations from Various Animal Sources*

	pH 3.90		pH 5.42		pH 6.40		pH 8.42		pH 10.65		pH 11.90		Mean per cent composition†
	d‡	r‡	d	r	d	r	d	r	d	r	d	r	
Beef													
C, ‡%	78.7	79.5			79.5	80.1	81.8	82.8					C = 81.1 ±0.52
I, ‡%	80.4	82.3			19.9	20.5	82.2	83.5					
	21.3	20.5					17.2	18.2					
	19.6	17.7					16.4	17.8					
C, cm. ² per volt sec. × 10 ⁻⁵	5.68	5.14	4.58	4.24	2.46	2.44	2.38	2.33	0.28	0.39	5.00	5.00	I = 18.9 ±0.52
I, " " × 10 ⁻⁵	5.68	5.76					2.35	2.30	0.24		4.34	4.25	
	0.56	0.63	0.38	0.36	1.79	1.66	2.41	2.20	2.80	2.61			
	0.61	0.74					2.50	2.22					
Horse													
C, % I, %	<div>←————— 100 —————→</div>												
	<div>←————— 0 —————→</div>												
C, cm. ² per volt sec. × 10 ⁻⁵	5.68	5.60	4.70	4.63	2.56	2.66	2.57	2.70	0.79	0.74	5.00	4.87	C = 100 ±0.32§
I, " " × 10 ⁻⁵	5.78	5.84					2.62	2.59	0.67	0.61			
Pig													
C, %	88.3	88.3			89.0	89.4	89.6	88.9			89.2	91.0	C = 89.3 ±0.26
I, %	11.7	11.7			10.6	11.0	90.1	89.5			10.8	9.0	
							11.1	10.5					
							10.6	9.9					
C, cm. ² per volt sec. × 10 ⁻⁵	5.81	5.45	4.58	4.05	2.35	2.40	2.12	1.93	0.25	0.32	5.28	5.32	I = 10.7 ±0.26
I, " " × 10 ⁻⁵	5.68	5.65					2.24	2.42			3.84	3.76	
	0.45		0.25	0.28	1.90	1.80	2.17	2.02	1.94	1.92			
	0.47						2.36	2.33					

Chicken

	pH 5.42	pH 6.48	pH 8.52	pH 10.15	pH 11.90	
<i>C</i> , %		88.0	89.4	88.5		<i>C</i> = 89.0 ±0.30
<i>I</i> , %		9.5	10.2	12.1		
<i>C</i> , cm. ² per volt sec. × 10 ⁻⁶			9.3	12.0		
<i>I</i> , " " " × 10 ⁻⁶			1.99	1.21		
	3.52	2.19	2.03	1.11	4.72	<i>I</i> = 10.9 ±0.36
	3.57		2.10	1.24	6.45	
	1.06	2.27	3.04	3.25	6.31	
			2.97	3.30		

* In several buffers, the mobility of one or the other of the migrating components in the mixture was so low as to resolve in- completely from the buffer anomalies in the duration of the run. In these instances, precise area determinations were not possible and these values are not included in the calculation of the means.

† Mean ± the standard error of *n* measurements.

‡ *C*, cytochrome *c* component; *I*, impurity; *d*, calculated from the descending patterns of the component; *τ*, from rising patterns.

§ The horse preparation showed no impurity and migrated as a single component over the entire pH range studied. Tiselius (12) has demonstrated that a protein concentration as low as 0.02 per cent may be detected by electrophoresis; hence, for com- parative purposes, the mean concentration was assumed to be in error by ±1 per cent, permitting the derivation of the statistic given in the table.

suppressed. Even under such favorable conditions, one or more small convection bands were occasionally seen, usually on the leading edges of the boundaries from either leg of the cell (note Figs. 2, 4, and 5).

Buffers for the beef, horse, and pig series were prepared as follows: acetate, pH 3.90 and 5.42; potassium phosphate, pH 6.40; sodium veronal-hydrogen chloride, pH 8.42; glycine, pH 10.65 and 11.90. For the chicken

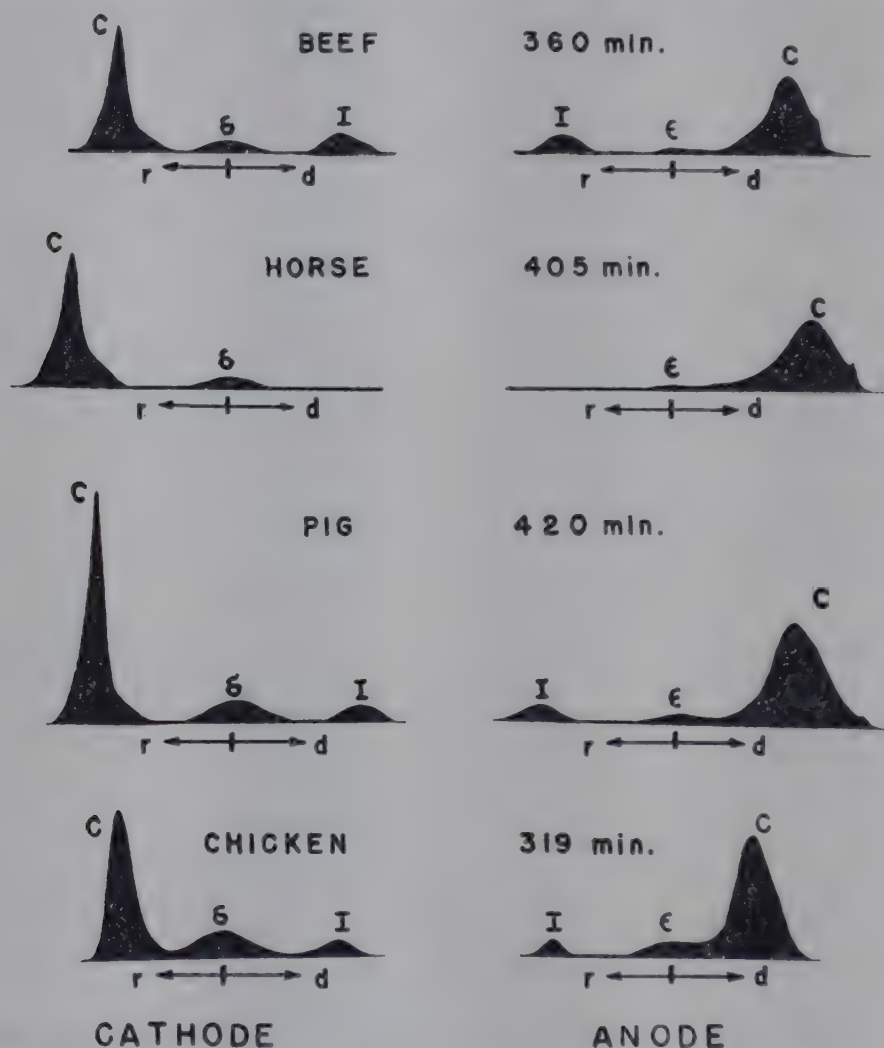


FIG. 2. Electrophoretic patterns of reduced cytochrome *c* preparations; protein concentration of each approximately 1.5 per cent. Veronal buffer, pH 8.42 (beef, horse, and pig) and 8.52 (chicken); ionic strength 0.2 (including 0.125 *N* NaCl); potential gradient, 4.0 volts per cm. C, cytochrome; I, impurity; stationary buffer boundaries, ϵ (descending side) and δ (rising side).

series the buffers were acetate, pH 5.42; phosphate, pH 6.48; veronal, pH 8.52; glycine, pH 10.15 and 11.90. All buffers except veronal were prepared at 0.1 ionic strength in buffer components and then were made up to 0.2 ionic strength with NaCl. Because of the lower solubility of veronal at the temperature of the electrophoretic bath, this buffer was prepared at 0.075 ionic strength, which was then increased to 0.2 with NaCl. The pH of each buffer was measured by the glass electrode at

several temperatures ranging from 10–25°, and its value at 0° was found from a plot of pH *versus* temperature.

Composition and mobility data were computed from the patterns from both legs of the cell; corrections for buffer anomalies according to the method of Longworth and MacInnes (9) were applied. The areas of the patterns were resolved either by the method of Pedersen (10) or of Longworth (8), depending upon the degree of separation of the components.

The usual procedure for calculating the percentage composition of a

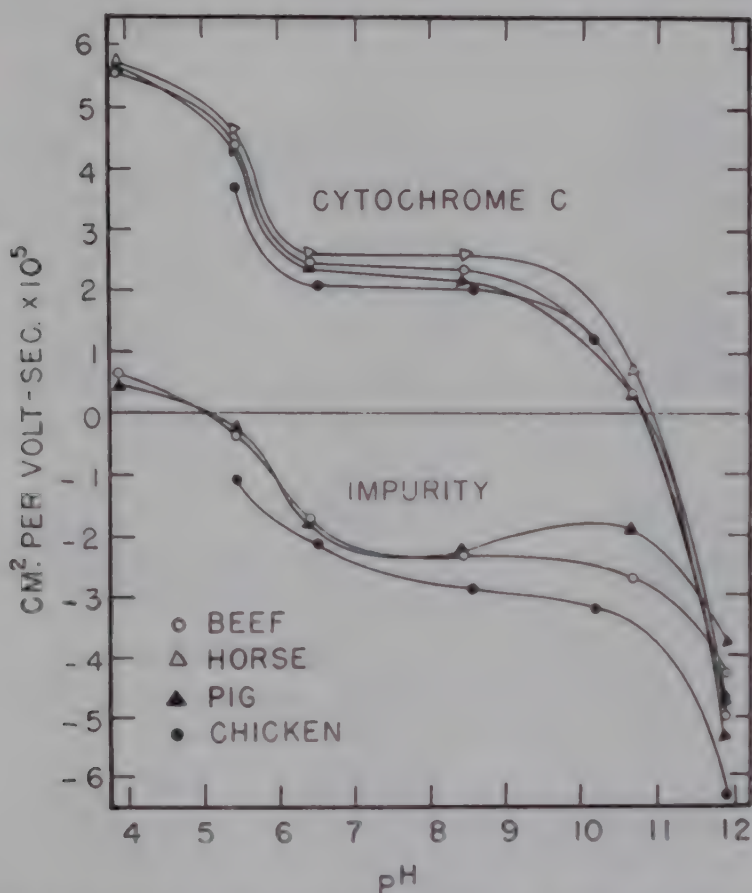


FIG. 3. Electric mobility curves of components in reduced cytochrome *c* preparations; ionic strength 0.2; temperature 0.5°. Cytochrome isoelectric in alkaline range, impurities in acid range. Migration toward the cathode positive; toward the anode, negative.

component has been in terms of the ratio of the area of the component to that of the total refractive index gradient on the particular side studied less that of the buffer anomaly. In the present instance, however, at several pH values the single impurity in each cytochrome preparation migrated in the opposite direction from the principal component in either leg of the cell. Thus, on either side a rising and a descending component was present. Since it has been demonstrated that, due to concentration changes, rising areas are generally smaller than corresponding descending ones (9), it was considered a more equitable procedure to calculate the

percentage composition of the component in terms of the sum of the two components migrating in the same direction on both sides of the cell.

The mobilities of each component were calculated from the displacement of the centroidal ordinate (11) of its refractive index gradient from the position of the original boundaries represented by the buffer anomalies, at one or more periods during the run.

Data for mobility of the several cytochrome *c* preparations are presented in Table I, and patterns obtained in the veronal buffer, representative of all the runs, are shown in Fig. 2. From the mean mobility values at each pH, electrophoretic mobility curves were plotted as shown in Fig. 3.

RESULTS AND DISCUSSION

All the mobility curves for the principal components, isoelectric in the pH range approximately 10.5 to 10.8, demonstrated the same general contour described for cytochrome *c* by Theorell and Åkesson (5). At any given pH, the absolute mobility differences among the preparations were of quite small magnitude; however, the persistent corresponding mobility differences over much of the pH range studied strongly suggested a significant type specificity. The preparation from horse heart demonstrated only a single migrating component in all the buffers, thus representing a presumably pure preparation. The beef, pig, and chicken preparations showed a single impurity. From the similarities in the mobility curves of these impurities, all isoelectric at approximately pH 5, there appeared to be the same or similar impurities in each of these preparations.

It is interesting to note that the mobility characteristics of the impurities generally resembled those of the albumins, particularly with respect to the practically uniform rate of mobility in the pH range 7 to 10. An albumin-like protein of the pressed muscle extract could conceivably remain in solution in the concentrated salt solution from which the cytochrome *c* is finally precipitated, and could thus be occluded in the final product. Such an impurity would tend to be diminished in concentration upon repetition of the extraction process. This condition is in accord with the observations of Drabkin (13) that, in a series of extractions of the pigment, the procedure which involved a repetition of the basic extraction method tended to yield the highest degree of refinement in the final product. In the present studies, by a similar extraction technique, the final products contained varying amounts of impurities, which were, however, of sufficiently low concentration to yield products of iron content in excess of 0.34 per cent, as confirmed in Paper II.

It is inconceivable that a uniform extraction, as performed in the pro-

duction of the several preparations, would lead to products of widely varying purities if a splitting of the protein portions of intracellular cytochrome *c* occurs, as suggested by Keilin and Hartree (6). Since the enzymatic activity of the products containing Fe in excess of 0.34 per cent was indistinguishable on an iron basis from that of preparations containing lower relative amounts of iron, it does not appear likely that a fragmentation of the molecule is involved. The present preparations, when tested with cytochrome oxidase from hog kidney (14) on a hydroquinone substrate in Warburg manometers at 25°, pH 7.1, exhibited uniform activity

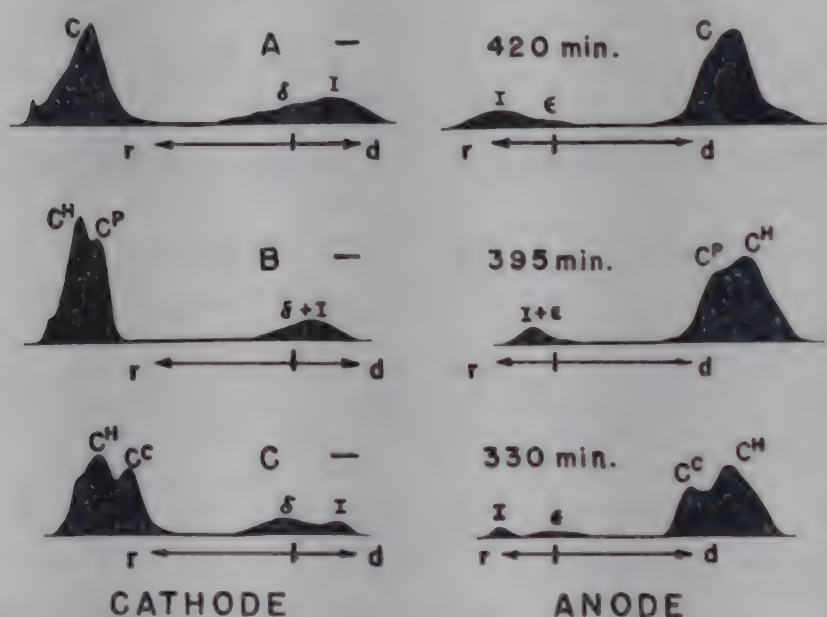


FIG. 4. Electrophoretic diagrams of mixed cytochrome *c* preparations reduced prior to dialysis. Total cytochrome *c* concentration of each, 1 per cent. Acetate buffer, pH 5.53; ionic strength 0.2 (including 0.1 *N* NaCl); potential gradient 4.0 volts per cm. Mixtures of equal parts (based upon pure cytochrome *c* content), A, beef plus chicken (represented as *C*); B, horse (*C^H*) plus pig (*C^P*); and C, horse (*C^H*) plus chicken (*C^C*).

per unit of iron (195 to 205 μ l. of O₂ per hour per microgram of cytochrome Fe). When tested on an equivalent weight basis, the isolated impurities in these preparations, separated by electrophoresis, were inactive.

Electrophoresis of Mixed Preparations

As a check on the specificity of these preparations, various mixtures were studied. In one series, combinations of two preparations were made containing equal amounts of each type before dialysis, computed from the purities established above. The patterns obtained in acetate buffer, pH 5.53 and 0.20 ionic strength, appear in Fig. 4. The resolution of components clearly paralleled the magnitude of the mobility spread previously established at this pH. The combination of the two preparations

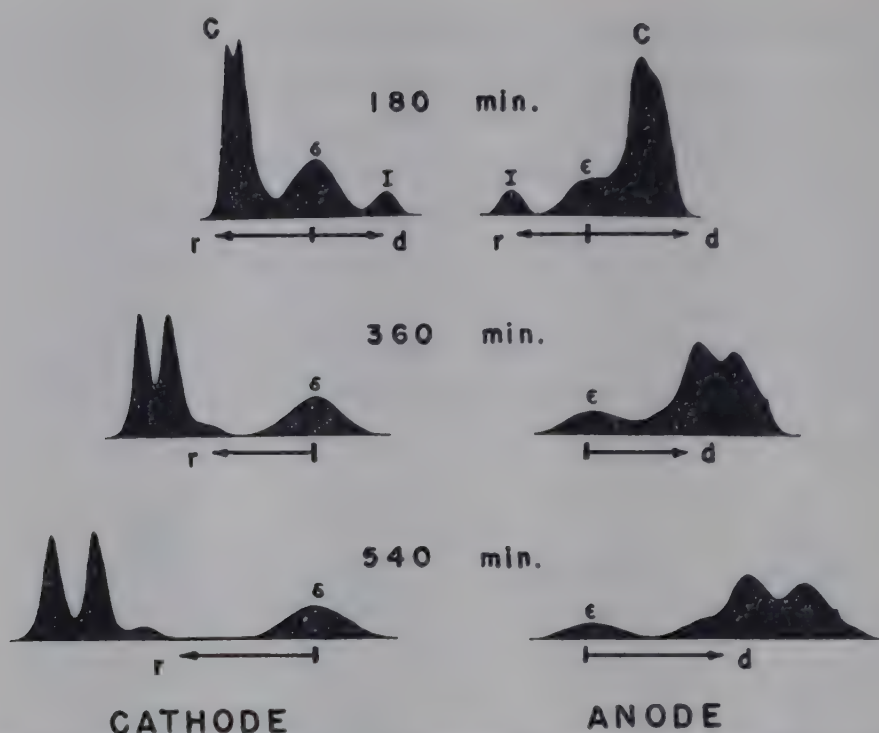


FIG. 5. Electrophoretic diagrams of a mixture of three cytochrome *c* preparations. Total concentration, 1.5 per cent on pure cytochrome basis, containing equal quantities of horse, pig, and chicken pigment. Veronal buffer, pH 8.48; ionic strength 0.15 (including 0.075 *N* NaCl); potential gradient 4.0 volts per cm. Impurities (*I*) permitted to migrate out of the cell.

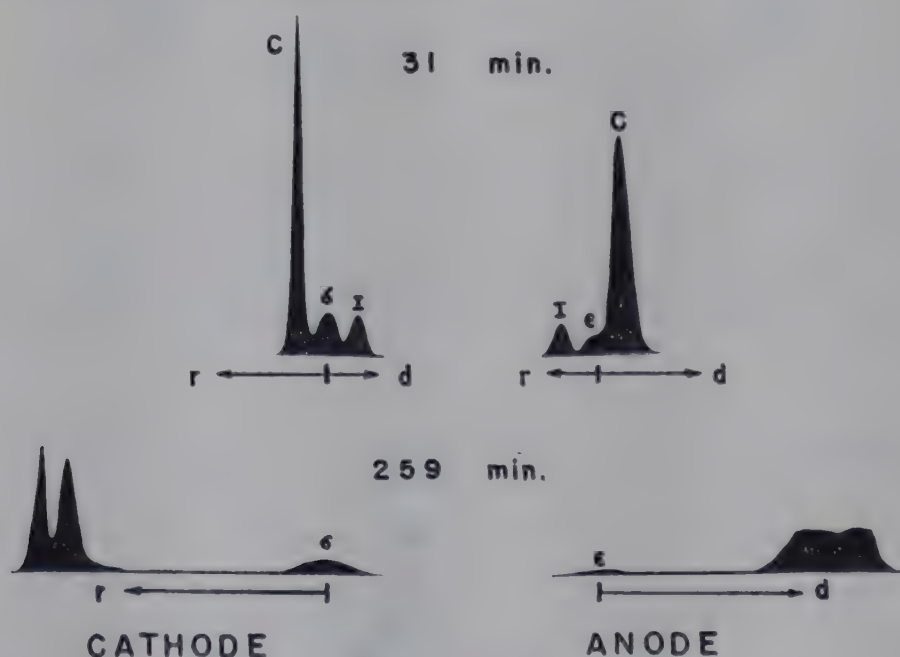


FIG. 6. Electrophoretic patterns of a mixture of equal portions (pure basis) of horse, beef, and pig cytochrome *c* preparations; total protein concentration approximately 1 per cent. Phosphate buffer, pH 5.45; 0.10 ionic strength; potential gradient 6.12 volts per cm.

most widely different in mobility, chicken and horse, demonstrated the most distinct separation into two well defined peaks in the shortest running time. Horse and pig cytochromes were moderately easily resolved. The

beef and chicken preparations gave evidence of component separation but resolution was relatively poor. None of the combinations dissociated in stoichiometric proportions.

The study of mixtures of these generic types was extended to three components as demonstrated in Figs. 5 and 6. In Fig. 5, horse, pig, and chicken cytochromes were combined, in equivalent amounts, at a total pigment concentration of 1.5 per cent, and run in veronal buffer, pH 8.48, 0.15 ionic strength; in Fig. 6, beef, pig, and horse cytochromes were combined at a total concentration of approximately 1 per cent and run in phosphate buffer, pH 5.45 and 0.1 ionic strength. In these instances again, the tendency toward resolution into numbers of peaks corresponding to the number of cytochromes making up the combination is evident; however, the departure from correspondence between relative areas and starting concentrations is more pronounced. The slowest component in Fig. 5 is markedly reduced in area, and in Fig. 6 it is practically obliterated, the two slower components having apparently merged into a single broader peak.

That these generic types of cytochrome *c* exhibit distinct specificity is thus apparent from a comparison of their relative electrophoretic mobilities when tested singly and as mixtures. Descriptions of similar specificity in proteins are not uncommon in the literature, for instance, hemoglobin (15), egg albumin (15), hemocyanin (16), etc. Whether or not the failure to obtain resolution of mixed cytochromes in stoichiometric proportions resulted from interactions among pigments of different genera is not evident from the limited data on the electrophoresis of mixed preparations.

SUMMARY

Cytochrome *c* preparations of heart muscle from four different animal sources, isolated by uniform salting-out procedures according to a modified standard method, showed varying degrees of purity by electrophoretic examination: beef, 81 per cent; horse, 100 per cent; pig, 89 per cent; and chicken, 89 per cent. The preparations lacking complete purity demonstrated single impurities whose electrophoretic behavior suggested their similarity in each case. The concentrated impurities showed no enzymatic activity. The relative electric mobilities of the principal components, tested in the reduced form over a wide pH range, were slightly but significantly different. This specificity for mobility differences was confirmed by the electrophoretic behavior of various mixtures of these preparations.

BIBLIOGRAPHY

1. Theorell, H., *Biochem. Z.*, **279**, 463 (1935).
2. Theorell, H., *Biochem. Z.*, **285**, 207 (1936).

3. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **122**, 298 (1937).
4. Theorell, H., and Åkesson, Å., *Science*, **90**, 67 (1939).
5. Theorell, H., and Åkesson, Å., *J. Am. Chem. Soc.*, **63**, 1804 (1941).
6. Keilin, D., and Hartree, E. F., *Biochem. J.*, **39**, 289 (1945).
7. Longworth, L. G., *Ind. and Eng. Chem., Anal. Ed.*, **18**, 219 (1946).
8. Longworth, L. G., *Chem. Rev.*, **30**, 323 (1942).
9. Longworth, L. G., and MacInnes, D. A., *J. Am. Chem. Soc.*, **62**, 705 (1940).
10. Svedberg, T., and Pedersen, K. O., *The ultracentrifuge*, Oxford, 293 (1940).
11. Longworth, L. G., *Ann. New York Acad. Sc.*, **41**, 267 (1941).
12. Tiselius, A., *Biochem. J.*, **31**, 1464 (1937).
13. Drabkin, D. L., *J. Biol. Chem.*, **140**, 373 (1941).
14. Haas, E., *J. Biol. Chem.*, **148**, 481 (1943).
15. Landsteiner, K., Longworth, L. G., and van der Scheer, J., *Science*, **88**, 83 (1938).
16. Tiselius, A., and Horsfall, F. L., Jr., *J. Exp. Med.*, **69**, 83 (1939).

STUDIES ON THE PURITY AND SPECIFICITY OF CYTOCHROME *c*

II. SPECTROPHOTOMETRIC CONSTANTS AND MOLECULAR WEIGHT DETERMINATION FROM THE CONTENT OF IRON

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In extractions of cytochrome *c* from various animal sources by a modified Keilin and Hartree procedure (1, 2), the iron content of the final products frequently exceeded an anticipated level of 0.34 per cent (2-4). This observation raised a question regarding the validity of the assumption that an iron content of 0.34 per cent is an evidence of complete purity. On the other hand, spectrophotometric determinations of purity, based upon molecular extinction coefficients derived by Rosenthal and Drabkin (5) (iron content 0.43 per cent (6, 7)), frequently yielded values in excess of 100 per cent. This fact, in turn, suggested the possibility that the iron content of the molecule may actually exceed 0.43 per cent, or that the coefficients are numerically low, or both.

It therefore seemed advisable to investigate further the relationship between iron content and purity. In Paper I in this series (8), the preparation of four lots of cytochrome *c* from several sources (beef, horse, pig, and chicken) was described as well as the determination of their purity by electrophoretic procedures. These data, in conjunction with iron determinations upon salt-free preparations, provided the basis for calculating absolute iron content, molecular weight, and other constants pertinent to spectrophotometric properties of cytochrome *c*, as subsequently described in this paper. A further objective, in view of the species specificity of the cytochrome preparations with respect to electrophoretic properties established earlier (8), was to investigate the possibility that such specificity may be expressed in other physical constants.

Preparation

The preparation of cytochrome *c* from heart muscle of beef, pig, horse, and chicken, together with the electrophoretic properties, has been described in Paper I (8).

For the determination of other physical properties of the pigments, the solutions of cytochrome *c* in saline (8) were dialyzed against repeated changes of distilled water made alkaline with dilute NH_4OH (1 part in

2500) until salt-free. This procedure was effective in suppressing the tendency of cytochrome *c* to penetrate cellophane sacs when dialyzed against distilled water (7). Traces of insoluble matter within the sacs were filtered, and the preparations were dried from the frozen state, whereby residual ammonia was driven off owing to the basic properties of the pigment. The dry preparations could be stored indefinitely at refrigerator or room temperature and could still be reconstituted to clear solutions with distilled water, suggesting little or no denaturation during drying or storage.

Iron Content

The usual procedures for the determination of iron in cytochrome *c*, and other similar organic complexes, have generally involved oxidation of the complex, followed by color development, and evaluation against a standard; *e.g.*, the method of Lorber (9) as used by Theorell and Åkesson (7), the dipyridyl method of Hill and Keilin (10, 11), titanous sulfate titration (12), and the *o*-phenanthroline method as adapted by Drabkin (13). Drabkin has made a critical study of several of these methods and has concluded that the *o*-phenanthroline procedure appears to be satisfactory for samples of hemin and cytochrome *c*. This method was therefore selected for routine use, and the relatively high degree of reported precision was verified by repeated tests.

Seven to nine preparations of different concentrations of cytochrome *c* from each species were analyzed by this method. All lyophilized preparations were dried to constant weight at room temperature under a vacuum over P_2O_5 , and then solutions were prepared with concentrations of the order of 5 to 10 mg. of cytochrome *c* per ml. in distilled water. Under the conditions of the test, the solutions were subsequently diluted to approximately 0.4 to 0.5 mg. per ml., at which level the color of the *o*-phenanthroline complex was read. The mean values and their precision indices for these determinations are presented in Table I. The data for purity as determined electrophoretically (8) are also included as a basis for determinations of absolute Fe and molecular weight.

Hemin iron was apparently present only in the cytochrome component of each preparation. Subsequent assays for Fe in concentrates of the impurities separated during electrophoresis revealed no detectable Fe content.

The data in Table I show that the cytochrome *c* preparations fall into two groups with respect to iron content and corresponding molecular weight. The horse and beef cytochrome represented an Fe content slightly, but significantly, higher than that of the chicken and pig, and had correspondingly different molecular weights. The differences be-

tween pig and chicken cytochrome were of questionable significance from the statistical examination of the results. It is noteworthy that the lower molecular weights of the horse and beef preparations were associated with corresponding higher electrophoretic mobilities (8) over most of the pH values investigated, and that the chicken preparation, with the highest

TABLE I

*Fe and Molecular Weight Determinations of Cytochrome c Preparations**

Type	Electrophoretic purity	Fe content		Molecular weight‡
		Direct determination	Pure basis†	
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
Beef	81.1 ± 0.52 (<i>n</i> = 10)	0.368 ± 0.0045 (<i>n</i> = 7)	0.453 ± 0.0063	12,350 ± 175
Horse	100.0 ± 0.32 (<i>n</i> = 10)	0.456 ± 0.0047 (<i>n</i> = 9)	0.456 ± 0.0049	12,270 ± 125
Pig	89.3 ± 0.26 (<i>n</i> = 10)	0.384 ± 0.0035 (<i>n</i> = 8)	0.430 ± 0.0042	13,000 ± 125
Chicken	89.0 ± 0.30 (<i>n</i> = 10)	0.374 ± 0.0018 (<i>n</i> = 8)	0.421 ± 0.0025	13,270 ± 80
$S_{\frac{1}{2}}$ L.S.D., <i>P</i>	0.05		±0.009 0.019	±254 520

* Values are means ± the standard error (S.E.) for *n* variates. The precision indices for means based upon direct readings are calculated as $\pm \sqrt{d^2/(n(n-1))}$. Those for calculated constants (absolute Fe content and molecular weight) are derived from the following relationship (14):

$$\frac{(\text{s.e. derived mean})^2}{(\text{derived mean})^2} = \frac{(\text{s.e. observed mean } X)^2}{(\text{observed mean } X)^2} + \frac{(\text{s.e. observed mean } Y)^2}{(\text{observed mean } Y)^2}$$

$$\dagger \text{ Absolute Fe} = \frac{\text{Fe determined}}{\% \text{ purity}} \times 100.$$

$$\ddagger \text{ Molecular weight} = \frac{55.84}{\% \text{ Fe, absolute}} \times 100.$$

§ $S_{\frac{1}{2}}$ = the standard error of the theoretical group mean from the pooled variance of the individual means. This statistic was employed for calculating the least significant difference (L.S.D.) between any two means at the probability level indicated for 28 degrees of freedom (15).

apparent molecular weight, generally possessed the lowest mobility in the same pH range.

The absolute values for Fe content of the horse and beef preparations were statistically similar, but both were significantly in excess of 0.43 per cent. The investigations of Roche *et al.* (16) upon salting-out procedures for cytochrome *c* from animal sources (beef and dog) have likewise indicated that the Fe concentration of the chromoprotein may exceed

this amount. Furthermore, direct molecular weight determinations upon cytochrome *c* by sedimentation equilibria and diffusion determinations have indicated molecular weights of 12,000 and 12,400 for the pigment (17). These values correspond to respective Fe contents of 0.465 and 0.45 per cent.

Theorell and Åkesson (7) found no differences in the physical properties of cytochrome *c* from beef and horse; however, based upon sulfur analyses in relation to the theoretical number of atoms per mole, they calculated a theoretical molecular weight of 13,120 and a hemin iron content of 0.425 per cent. Because of this, their directly determined Fe content of 0.447 per cent, a value more consistent with the above findings, was corrected downward to the lower level on the assumption of the presence of contaminating non-cytochrome iron. They describe other preparations, however, of considerably lower purity (0.69 and 0.74), extracted originally by the same basic procedure without electrophoretic purification, but containing no iron other than that of cytochrome. In the present experiments, similarly, no traces of non-cytochrome iron were evident in any of the impurities of the preparations.

Spectrophotometry

Each of the cytochrome *c* preparations of the present study showed the characteristic absorption maxima (417, 521, and 550 $m\mu$) when in the fully reduced state (1, 4, 5, 18–20). Although the pigments dissolve readily to form optically clear solutions, experience has indicated that optical density readings made immediately following solution are often 10 to 15 per cent lower than those made when reduction is carried out at least 30 minutes after solution of the pigment is effected. After this period, the solutions are apparently at equilibrium, since no further increases in optical density are observed with freshly reduced samples.

Aliquots of each salt-free lyophilized preparation, dried to constant weight *in vacuo* at room temperature, were dissolved in 0.1 M acetate buffer, pH 6.0, in concentrations varying from 0.1 to 0.3 mg. per ml. of solids. All solutions could be reconstituted without any insoluble residue or visible turbidity. After a minimum period of 30 minutes, spectrophotometry with 1 cm. cuvettes was carried out in the usual manner, following reduction of the pigment with solid sodium dithionite. The reducing agent was added directly to the solution in the cuvette in the approximate amount of 0.1 mg. per ml.

The optical densities of these solutions were read principally in the range of the maximum band at 550 $m\mu$ and the minimum absorption level at 535 $m\mu$. From these density values, the extinction coefficients for the preparations at concentrations of 1 gm. of total solids per 100 ml. of

TABLE II
Spectrophotometric Constants for Cytochrome *c* Preparations upon Fe Basis*

Preparation	$E_1^{1\%}$ 550 $m\mu$		$\epsilon_{550} m\mu^\dagger$	$E_1^{1\%}$ 535 $m\mu$		$\epsilon_{535} m\mu^\dagger$	$\frac{\epsilon_{550} m\mu}{\epsilon_{535} m\mu}$
	Measured value	Corrected for purity		Measured value	Corrected for purity		
Beef	17.34 \pm 0.25 ($n = 7$)	21.38 \pm 0.34	26,400 \pm 560	4.62 \pm 0.06 ($n = 7$)	5.69 \pm 0.08	7030 \pm 140	3.76 \pm 0.11
Horse	22.73 \pm 0.43 ($n = 7$)	22.73 \pm 0.44	27,840 \pm 600	5.89 \pm 0.12 ($n = 7$)	5.89 \pm 0.13	7230 \pm 170	3.83 \pm 0.13
Pig	19.28 \pm 0.25 ($n = 7$)	21.58 \pm 0.29	28,030 \pm 460	5.04 \pm 0.06 ($n = 7$)	5.65 \pm 0.07	7340 \pm 160	3.82 \pm 0.10
Chicken	18.10 \pm 0.15 ($n = 6$)	20.34 \pm 0.18	26,980 \pm 290	4.79 \pm 0.03 ($n = 6$)	5.38 \pm 0.04	7150 \pm 70	3.77 \pm 0.05
S_z^\ddagger		± 0.69	$\pm 1,020$		± 0.17	± 290	± 0.21
L.S.D., P	0.05	1.43	2,110		0.36	600	0.44
" "	0.10	1.18	1,750		0.30	500	0.37

* Derived and propagated means \pm standard errors.

$^\dagger \epsilon$ ($c = 1$ mole per liter, based on the Fe determinations and the molecular weights corrected for electrophoretic purity, $d = 1$ cm.). Corresponding values (ϵ mM, based upon 0.43 per cent Fe) calculated from Theorell's (4) absorption coefficients by Drabkin (20) are 28.10 at 550 $m\mu$ and 10.22 at 540 $m\mu$, yielding the low ratio of 2.75. Drabkin presents values on ferrocytochrome *c* solutions, pH 4.10 to 4.92, of 26.11 at 550 $m\mu$ and 7.39 at 535 $m\mu$; ratio 3.53. In solutions equivalent to 0.2 N NaOH (5, 20), the extinction constants at 550 $m\mu$ increased numerically to 28.38 and 29.28, with corresponding values at 535 $m\mu$ of 7.29 and 9.45; ratios 3.89 and 3.10 respectively. The higher extinctions at 550 $m\mu$ and the higher ratio are more consistent with the present findings, determined, however at pH 6.0.

$^\ddagger S_z$ = the standard error of the theoretical group mean from the pooled variance of the individual means; L.S.D., the least significant difference among the means at the probability levels, $P = 0.05$ and 0.10, 23 degrees of freedom (15).

the solution were calculated ($E_{1\text{ cm.}}^{1\%}$, 550 $m\mu$ and $E_{1\text{ cm.}}^{1\%}$, 535 $m\mu$). These apparent values were then corrected for the purity established electrophoretically. Finally, by employing values for the molecular weights derived earlier from the Fe content, molecular extinction coefficients for these wave-lengths, ϵ ($c = 1$ mole per liter, $d = 1$ cm.), were calculated. These data are presented in Table II.

Spectrophotometric evaluation of the concentrated impurities, isolated in the electrophoresis apparatus, tested at concentrations of the order 1 to 5 mg. per ml., and thus approximately 10 times the order of magnitude employed in the analyses of the entire preparations, yielded densities of 0.024 to 0.035 over the visible spectrum range. The preparations as a whole, in the smaller concentrations tested, yielded values ranging from 0.300 to 0.400 for the maximum band and 0.090 to 0.120 for the minimum band. Since the relative concentrations of the impurities in the several preparations ranged from 0 to approximately 20 per cent of the total solids, it was obvious that the base-line absorption of the impurities in the preparations as tested was negligible. That these impurities could be considered relatively spectroscopically inert was borne out by the fact that the ratio of the two molecular extinction values remained constant from the horse preparation, with no evident impurity, to that of the beef with 19 per cent impurity. Base-line absorption which increased extinction at both wave-lengths by equal amounts could be expected to alter these ratios.

It is of interest to note from Table II that the species specificity indicated by the Fe content and the molecular weight of the several preparations is also evident in the spectrophotometric constants governed by weight relationships ($E_{550\text{ m}\mu}^{1\%}$ and $E_{535\text{ m}\mu}^{1\%}$). However, when molecular extinction coefficients were calculated, based upon corresponding derived molecular weights for each preparation, these constants ($\epsilon_{550\text{ m}\mu}$ and $\epsilon_{535\text{ m}\mu}$) showed no statistically significant differences. This fact, coupled with the uniformity of the extinction ratios, suggested that the specificity of these preparations was a function of the protein portions of the molecules, rather than of the prosthetic groups, the latter apparently possessing remarkable uniformity.

SUMMARY

Determinations of iron upon salt-free preparations of cytochrome *c* from heart muscle of four different animal species, corrected for the purities of the preparations established by electrophoresis, showed the following percentage composition: beef, 0.453; horse, 0.456; pig, 0.430; and chicken, 0.421. Precision indices derived from the variance of the replicate determinations indicate that some of these calculated values are significantly

different. The values for horse and beef cytochrome *c* were significantly higher than the commonly accepted value of 0.43 per cent for cytochrome *c* and likewise higher than the Fe values for the pig and chicken preparations. When the Fe values were employed to calculate the molecular weights of the several cytochrome *c* preparations, corresponding significant differences were obtained: beef, 12,350; horse, 12,270; pig, 13,000; and chicken, 13,270.

Spectrophotometric constants for these pigments have been established. Extinction values at two wave-lengths upon a weight basis ($E_{1\text{ cm.}}^{1\%}$ 550 $m\mu$ and $E_{1\text{ cm.}}^{1\%}$ 535 $m\mu$) are significantly different; however, upon a molecular basis, by applying the corresponding molecular weights derived from the Fe determinations, the ϵ values ($c = 1$ mole per liter) are constant for all the preparations within normal probability levels, as are their ratios. This condition suggests the identity of the prosthetic group of the several pigments and differences among the protein portions of the molecules to account for the species specificity observed.

The authors wish to express their appreciation to Dr. D. L. Drabkin for valuable suggestions on certain phases of these investigations.

BIBLIOGRAPHY

1. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **122**, 298 (1937).
2. Keilin, D., and Hartree, E. F., *Biochem. J.*, **39**, 289 (1945).
3. Theorell, H., *Biochem. Z.*, **279**, 463 (1935).
4. Theorell, H., *Biochem. Z.*, **285**, 207 (1936).
5. Rosenthal, O., and Drabkin, D. L., *J. Biol. Chem.*, **149**, 437 (1943).
6. Theorell, H., and Åkesson, Å., *Science*, **90**, 67 (1939).
7. Theorell, H., and Åkesson, Å., *J. Am. Chem. Soc.*, **63**, 1804 (1941).
8. Tint, H., and Reiss, W., *J. Biol. Chem.*, **182**, 385 (1950).
9. Lorber, L., *Biochem. Z.*, **181**, 391 (1927).
10. Hill, R., and Keilin, D., *Proc. Roy. Soc. London, Series B*, **114**, 104 (1933).
11. Hill, R., *Proc. Roy. Soc. London, Series B*, **107**, 205 (1930).
12. McFarlane, W. D., *Biochem. J.*, **26**, 1034 (1932).
13. Drabkin, D. L., *J. Biol. Chem.*, **140**, 387 (1941).
14. Worthing, A. G., and Geffner, J., *Treatment of experimental data*, New York, 207 (1943).
15. Snedecor, G. W., *Statistical methods*, Ames, 4th edition, 80 (1946).
16. Roche, J., Derrien, Y., and Cahnmann, J., *Compt. rend. Soc. biol.*, **140**, 146 (1946).
17. Pedersen, K. O., and Andersson, K. J. I., in *Alexander's Colloid chemistry*, New York, **5**, 564 (1944).
18. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **129**, 167 (1940).
19. Theorell, H., *Biochem. Z.*, **298**, 202 (1938).
20. Drabkin, D. L., *J. Biol. Chem.*, **140**, 373 (1941).

MECHANISM OF FAT ABSORPTION AS EVIDENCED BY CHYLOMICROGRAPHIC STUDIES

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Frazer (1) has presented a large number of experimental findings to support his partition hypothesis, involving the particulate absorption of triglycerides, as opposed to the current lipolytic hypothesis as stated by Verzar and McDougall (2). The partition hypothesis is based upon experiments in which three different methods have been used: histological studies, chylomicrographic investigations of the absorption of triglycerides and free fatty acids, and absorption experiments with finely divided emulsions of paraffin oil. The failure of Lundback and Maaloe (3) to demonstrate the absorption of paraffin emulsions similar in particle size to those used by Frazer *et al.* (4) and our interest in fat absorption prompted us to attempt to obtain further evidence from chylomicrographic studies.

The present investigation was planned to furnish data concerning several general points related to the mechanism of fat absorption. More information was desired regarding the possible route of fat and fatty acids in the animal body, the ability to absorb unhydrolyzable substances in the presence of a natural emulsifying system, and the effect of choline on the rate of fat absorption.

Preliminary experiments had shown no difference in the number of fat particles in the systemic blood during the absorption of ingested triglycerides or their equivalent of free fatty acids and glycerol, as previously reported by Frazer (5). It was deemed best to reestablish first that the number of chylomicrons could be used as a relative measure of the amount of fat absorbed, and then by this means to attempt to learn whether the free fatty acids followed a different pathway from that of the triglycerides.

Frazer *et al.* (4) found only one system, that of fatty acid-bile salt-monoglycerides, which permitted fine and stable emulsions of fat in the intestine. Two of these components were believed to be supplied by hydrolysis of only 30 per cent of the fat. The question arose as to whether mineral oil would be emulsified and absorbed if such a mixture of it with fatty acids and monoglycerides were ingested. A similar mixture of the latter substances with a didecyl ether of glycerol was also tested for a similar reason. Lack of absorption of either of these substances would not detract from the partition hypothesis, but their appearance in the blood would be evidence in its favor.

Phosphorylation with lecithin formation has been proposed as an important intermediate step in triglyceride resynthesis (6, 2). However, Frazer (1) regards lecithin as an end-product rather than an intermediate compound in resynthesis. Several investigators (7, 8) have shown that choline affects the rate of phospholipid turnover, and Frazer (9) has found that it influences the passage of fat through the intestinal mucosa. If choline is a limiting factor (7) in the formation of the phosphatides and if the latter are required for resynthesis of the triglycerides, choline should increase the rate of absorption when given with the fat. A comparison of the rates of absorption after giving the fat with lecithin and with choline might aid in answering a query raised by Augur *et al.* (10). They suggested the possibility that lecithin might promote fat absorption by increasing the speed and degree of emulsification, which would aid either hydrolysis or particulate absorption. Choline given parenterally could not aid this emulsification.

The results of the present experiments appear to support the lipolytic hypothesis.

EXPERIMENTAL

The effect of three levels of ingested fat, with or without added lecithin, on the relative fat content of the blood was demonstrated by chylomicron counts. Adult male albino rats were fasted for 24 hours and given either 0.04, 0.08, 0.10, or 0.12 ml. of olive oil or olive oil with 20 per cent egg lecithin per sq. dm. of body surface. These small quantities of fat were selected in an attempt to have the amount in the stomach as near physiological conditions as possible. Usually the total amount was 0.15 to 0.40 ml. per rat, with a maximum amount of 0.54 ml. However, in order to repeat a previous study (5), 1 ml. of fat was given in a part of one experiment (Table II). The fat was measured in a tuberculin syringe and introduced into the stomach by tube. Blood was obtained from the clipped tail of the fasted rat at intervals of 1, 1½, 2, and 3 hours after giving the fat. The same general procedure as that of Frazer *et al.* (11), with the modifications of Cooper and Lusk (12), was used for counting the chylomicrons. Counts on the blood of the fasting rats were made on the serum; all the others were made on 1:10 dilutions of whole blood. Dilutions were made with distilled water (13) in a Thoma white cell dilution pipette. The fat particles in twenty-eight large squares of a Whipple disk were counted for seven different fields, and an average count of ten of these squares was the value used.

This same procedure was followed throughout this study for determining the effect of the ingestion of the various substances on the level of blood fat. The weight of the fatty acids given in each experiment was accurately

determined from the volumes measured and so recorded. Surface areas were calculated by the formula of Rubner (14). The relative amounts of fat added to the blood during the absorptive periods, as well as the comparative rates of the processes (15), were estimated by determining the areas under the curves during the 1 to 3 hour period.

A comparison of the fat content of the systemic blood after the ingestion of a fat was made with that found after administration of an equivalent amount of fatty acids and glycerol. The rats were given either 0.04 or 1 ml. of olive oil per sq. dm. of body surface. On the same basis, 0.04, 0.12, and 1 ml. of fatty acids were administered, along with the amount of glycerol necessary to convert these acids into triglycerides. The glycerol was made up to 1 ml. with water and given through another tube immediately after the fat introduction.

Unexpected results with the rats caused the repetition of the experiment with human subjects; 30 gm. of olive oil and then 30 gm. of the fatty acids with an equivalent amount of glycerol were administered to each of ten men. This duplicated an experiment by Frazer (16), except that he gave lipase with the fat instead of glycerol with fatty acids. All fatty acids used in these studies were prepared from olive oil by the usual method. Precautions were taken to prevent oxidation of the unsaturated fatty acids during and after their preparation. The chylomicron counts were made on the blood after an overnight fast and 2, 2½, and 3 hours after ingestion of the fat or fatty acids and glycerol.

In the partition hypothesis it is held that about two-thirds of the unsplit fat is absorbed as such, aided by the emulsifying action of the remaining third, which is split to fatty acids and monoglycerides. A mixture of 2 moles of fatty acid to 1 mole of monoglyceride was dissolved in proportions of one-third in either mineral oil or a di-*n*-decyl ether of glycerol. These solutions would provide the necessary emulsifying system, similar to that which would be present after one-third of a fat had been hydrolyzed, and would possibly allow such a state of division in the intestine as to permit particulate absorption. The chylomicron levels in the blood were determined after the ingestion of fatty acids, of the equivalent of these fatty acids in the monoglyceride-fatty acid mixture, and of solutions of this mixture in mineral oil and the diether. The monoglyceride alone was given at three levels: 0.04 ml. (approximately equivalent in fatty acids to that of the above mixtures), 0.08 ml., and 0.12 ml. per sq. dm. of body surface.

The monoglyceride was prepared by heating mole for mole of glycerol and fatty acids at 240° while a stream of carbon dioxide was slowly passed through the mixture until after the two layers had disappeared. No free fatty acids remained and the acetyl number attested the presence of the

equivalent of two free hydroxyl groups. The di-*n*-decyl ether of glycerol was prepared by the method of Boyd and Marle (17) and it was proved to have only one free hydroxyl group per molecule.

The effect of choline upon the absorption of olive oil was determined on the fasted rats after the stock diet and after 7 days on a low choline, low fat diet (7). The same amount of olive oil was given to both groups, with and without the choline. The 30 mg. of choline chloride were dissolved in 1 ml. of water and given either orally or intraperitoneally. The area under the curve from the chylomicron counts was again used as a relative measure of the fat absorbed.

TABLE I

Relation of Chylomicron Counts to Fat Ingested

The rats were fasted 24 hours. The olive oil plain, or mixed with 20 per cent lecithin, was given by stomach tube from a tuberculin syringe. The chylomicron counts were made on blood expressed from the rat's clipped tail.

No. of rats	Fasting weight	Fat given	Fatty acids per sq. dm.	Chylomicron count					Area* under curve, 1-3 hrs.
				Fast-ing	1 hr.	1½ hrs.	2 hrs.	3 hrs.	
	<i>gm.</i>		<i>mg.</i>						
10	230	Olive oil	35	14	41	86	57	39	116 ± 3
10	258	" "	69	13	57	96	84	53	152 ± 6
10	256	" "	104	16	69	93	115	88	195 ± 5
8	298	" " + lecithin	34	15	59	107	67	54	147 ± 2
8	288	" " + "	67	15	68	123	92	71	184 ± 2
3	212	" " + "	83	19	79	96	136	61	201 ± 1

* Including the standard error of the mean.

Apparent differences were tested for significance by the *t* method of Fisher (18), and only those showing a *P* value of less than 0.01 were considered significant.

Results

Other investigators (15, 13, 12) have established that the chylomicrograph can be used as a measure of the relative amount of neutral fat in the blood, and that the area under the curve gives a similar measure of the fat in the blood during that period and the rate of the various processes involved. The data in Table I show that this area did increase with increased intakes of the fat within the range of the equivalents of 35 to 100 mg. of fatty acids per sq. dm. of body surface. The same was true of the fat containing 20 per cent lecithin, but the blood fat level was about 24 per cent higher than with the fat alone. In this range of intakes, a straight

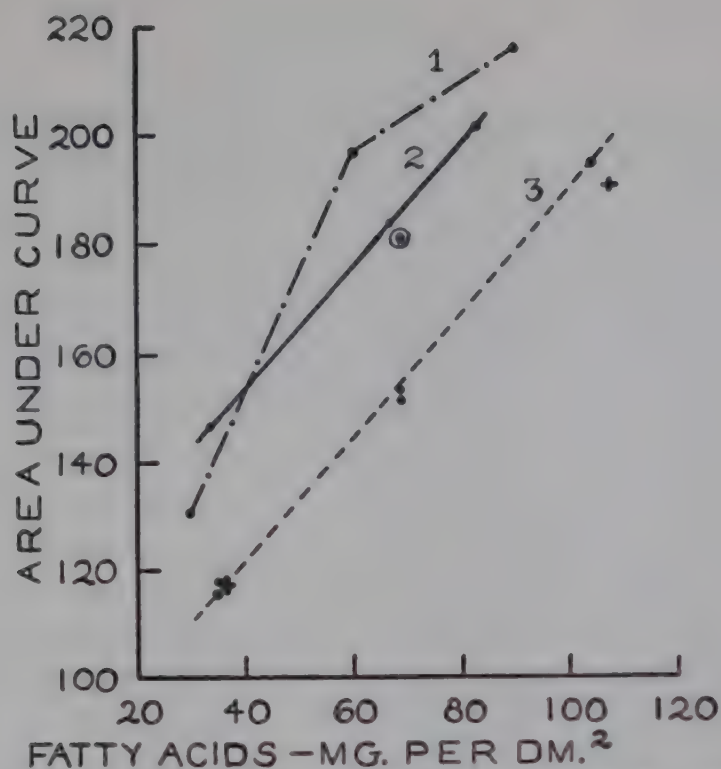


FIG. 1. Relation of the fatty acids to the area under the curve of the chylomicrograph as a relative measure of the fat absorbed. Curve 1, monoglyceride alone; Curve 2, olive oil with lecithin; Curve 3, olive oil; \odot , olive oil with choline, +, free fatty acids and glycerol.

TABLE II

Chylomicrographs after Ingestion of Fatty Acids and Triglycerides

The rats were fasted 24 hours and the human subjects 14 hours before ingestion of the olive oil or free fatty acid prepared from it. Glycerol equivalent to that of the neutral fat was given with the fatty acids. Chylomicron counts were made on blood from the tail or finger tip.

No. of rats	Fasting weight	Fat given	Fatty acids per sq. dm.	Chylomicron count					Area under curve, 1-3 hrs.
				Fasting	1 hr.	1½ hrs.	2 hrs.	3 hrs.	
	gm.		mg.						
8	318	Olive oil	35	14	53	91	53	39	118 ± 1
10	251	Fatty acids	36	15	54	91	52	40	118 ± 2
7	279	" "	107	16	66	87	125	75	191 ± 2
7	324	" "	206	16	73	102	134	74	207 ± 2
5	288	Olive oil	218	13	76	103	137	81	214 ± 2
3	286	" "	0	11	8	9	9	9	18 ± 2

Human subjects*

	kg.		gm.		2 hrs.	2½ hrs.	3 hrs.	2-3 hrs.
10	71	Olive oil	30	18	82	102	121	102 ± 1
10	71	Fatty acids	30	17	81	102	122	102 ± 1

* Medical students served as subjects in these last two groups.

line was obtained (Fig. 1) for both if the blood fat (area under the curve) was plotted against the total fatty acids given.

No significant differences were found in either the peaks of the chylomicrograph or the areas under the curves when comparable amounts of neutral fat and free fatty acids and glycerol were given. In Table II, the data obtained from three different levels of intake are listed for comparison. Similar results were obtained when human subjects ingested 30

TABLE III

Effect of Monoglyceride-Fatty Acid Mixtures on Blood Fat

The fatty acids from olive oil and their equivalents in neutral fat or in a monoglyceride mixture with either mineral oil or a diether of glycerol were given rats fasted 24 hours. The relative fat content of the blood was determined from chylomicron counts on blood from the tail. The monoglyceride was given alone at three different levels of intake.

No. of rats	Fasting weight	Fat given	Fatty acids per sq. dm.	Chylomicron count					Area under curve, 1-3 hrs.
				Fast-ing	1 hr.	1½ hrs.	2 hrs.	3 hrs.	
	gm.		mg.						
8	343	Fatty acids	36	15	30	59	74	49	117 ± 2
8	322	Mixture*	33	11	28	55	80	50	120 ± 3
8	320	“ + mineral oil	33	10	30	55	81	52	122 ± 3
8	315	Mixture + diether†	33	16	24	52	68	48	107 ± 1
8	334	Mineral oil	0	12	15	5	7	8	16 ± 1
8	318	Olive oil	35	14	53	91	53	39	118 ± 1
10	267	Mixture	33	13	54	89	53	38	118 ± 1
12	275	“ + diether	33	14	46	84	50	36	110 ± 1
4	279	Monoglyceride	30	11	59	92	65	42	131 ± 4
4	213	“	60	15	71	126	114	61	197 ± 3
4	306	“	89	15	81	123	126	80	216 ± 3

* The mixture contained 2 moles of fatty acid to 1 mole of monoglyceride. All fatty acids were prepared from olive oil.

† Glycerol di-*n*-decyl ether.

gm. of olive oil and then, several days later, 30 gm. of the fatty acids with an equivalent amount of glycerol.

The addition of the emulsifying system, a fatty acid-monoglyceride mixture, to twice its weight of mineral oil or a diether of glycerol did not increase the number of chylomicrons in the blood after these mixtures were ingested (Table III). The blood fat was affected only by the amount of fatty acids taken, except that the blood fat was significantly lower after ingestion of the diether-mixture and much higher after that of the monoglyceride alone. Lower values with the diether-mixture were confirmed by repeating that part of the experiment.

Choline, given either orally or intraperitoneally, was found to increase the blood fat level of the fat-fed animals that had been on either a normal or a choline-deficient diet above the level of those given fat alone (Table IV). These elevated levels were not significantly different from each other after either method of choline administration, or from that of the animals given a similar amount of fatty acids in the form of a fat with added lecithin. However, a similar amount of fatty acids given in the form of a monoglyceride was usually associated with significantly higher blood fat levels than those found after the fat with either lecithin or cho-

TABLE IV

Effect of Choline and Lecithin upon Fat Absorption

The olive oil was given by tube either with or without 30 mg. of choline in 1 ml. of water (orally or intraperitoneally) to rats fasted 24 hours. The lecithin was 20 per cent egg lecithin made up in olive oil. This was repeated in part after 7 days on a choline-deficient diet.

No. of rats	Fasting weight	Fat given	Fatty acids per sq. dm.	Chylomicron count					Area under curve, 1-3 hrs.
				Fast-ing	1 hr.	1½ hrs.	2 hrs.	3 hrs.	
12	gm.		mg.						
12	225	Olive oil	69	14	58	100	85	50	154 ± 5
12	223	" " + choline orally	69	13	67	102	107	52	174 ± 5
11	232	Same intraperi- toneally	69	13	71	106	107	60	181 ± 4
8	288	Lecithin	67	15	68	123	92	71	184 ± 3

After 7 days on choline-deficient diet

10	258	Olive oil	69	14	60	87	73	48	138 ± 2
10	263	" " + choline	69	13	66	97	102	64	174 ± 4

line. It is to be noted that the fat, when given alone, does not appear in the blood as rapidly after a choline-deficient diet as after a normal one.

DISCUSSION

The fat absorbed, as measured by the number of chylomicrons, appears to be related linearly, within the range studied, to the amount of fat administered, if it is given in proportion to the body surface. The uniformity of the results in all these experiments in which the body weight ranged from 200 to 350 gm. supports the finding of Deuel *et al.* (19) that most uniform results are obtained when the fat absorption is based on body surface.

Additional evidence to support the contention that the area under the

curve of the chylomicrograph is a fair measure of the amount of fat absorbed was obtained from the study of Augur *et al.* (10). By recovering the unabsorbed fat from the intestine after a 2 hour period, these investigators found that approximately 27 per cent more fat had been absorbed when the fat was given with lecithin. In the present study, a corresponding figure of 24 per cent increase in fat absorption was obtained in a similar experiment by comparing the corrected areas under the curve.

In repeated experiments, similar areas under the curve and similar peaks were obtained from the chylomicrographs after equivalent quantities of neutral fat and free fatty acids were given. The same amounts of these substances, 1 ml. to rats and 30 gm. to men, as given by Frazer and co-workers (5, 16), failed to show a difference in the chylomicrons of the systemic blood after ingestion of triglycerides or fatty acids. No explanation is available for this lack of agreement in results from that laboratory and this one. However, these findings do not support the contention that different pathways from the intestine are involved in the absorption of hydrolyzed and unhydrolyzed fat.

It is quite possible that the emulsifying system, fatty acids with monoglyceride, would not produce an emulsion in the intestine with mineral oil or the diether of glycerol similar to that with a triglyceride. The diether was used as an unhydrolyzable substance with a structure more like that of a diglyceride, and unlike the mineral oil, the one free hydroxyl group would permit esterification or phosphorylation. Substances of this nature have been reported to be constituents of liver oils of some marine fish (20). The mineral oil and diether mixtures were given on the chance that they might be emulsified, absorbed, and appear in the blood under these conditions, and, if so, support the partition hypothesis. However, the mineral oil and diether not only did not appear in the blood, but the diether significantly decreased the appearance of some of the fatty acids given with it, as shown in Table III. Lack of information on its effect on the absorptive processes permits only the guess that the diether was esterified in part and that this prevented the absorption of the fraction of the esterified fatty acids that would otherwise have been absorbed and converted to triglycerides.

Both the lecithin-fat mixture and the monoglyceride alone appeared to be absorbed at a faster rate than that of the fat or the fatty acids. Augur *et al.* (10) explained the increased rate of fat absorption when fat was given with lecithin as a possible effect of an increased emulsification, permitting either an increased rate of hydrolysis or particulate absorption. The increased rate after the monoglyceride ingestion supports the suggestion that an increased emulsification plays an important part. However, the

high rate of absorption of the fat with choline, given either orally or intraperitoneally, when an increased emulsification could not be involved, suggests a still more important rôle for the lecithin.

The decreased fat absorption after choline-deficient diets and the increased absorption associated with a choline supplement, given to rats on both normal and choline-deficient diets, are findings in complete agreement with those of Artom and Cornatzer (7). They reported that choline stimulated the phosphorylation of lipides in the intestine and suggested that choline may represent a limiting factor in the formation of phospholipides during the absorption of fat from the intestine.

Thus if choline affects the rate of phospholipide turnover, is a limiting factor in formation of intestinal phosphatides, and increases the rate of the passage of fat through the intestinal mucosa, then it would appear to have a more important rôle than that of an end-product in the process of fat absorption. The increased rate of absorption with added lecithin or choline seems more likely explained, in part at least, by the presence of the phosphatide, or the choline for its synthesis, needed for an important intermediate step in the resynthesis of triglycerides.

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SUMMARY

The absorption of neutral fat, or the free fatty acids prepared from it, gave a similar systemic lipemia. This was true at three different levels of fatty acid ingestion in the rat and at one level in man. This does not agree with the suggestion of different pathways for these substances after absorption.

An emulsifying system, fatty acids with monoglyceride, did not cause an increased number of chylomicrons in the blood when ingested with either mineral oil or a diether of glycerol. Instead, the latter depressed the number of fat particles that would have been present from fatty acids ingested with it.

An increased rate of fat absorption did follow the ingestion of fat with added lecithin or of monoglyceride alone. The fact that choline given along with the fat had a similar effect suggests that this increased rate of absorption cannot be exclusively the result of an increased emulsification of the fat in the intestine, but must involve choline in some other important rôle.

BIBLIOGRAPHY

1. Frazer, A. C., *Physiol. Rev.*, **26**, 103 (1946).
2. Verzar, F., and McDougall, E. J., Absorption from the intestine, London (1936).
3. Lundbaek, K., and Maaløe, O., *Acta physiol. Scand.*, **13**, 247 (1947).
4. Frazer, A. C., Schulman, J. H., and Stewart, H. C., *J. Physiol.*, **103**, 306 (1944).
5. Frazer, A. C., *J. Physiol.*, **102**, 306 (1943).
6. Sinclair, R. G., *J. Biol. Chem.*, **82**, 117 (1929).
7. Artom, C., and Cornatzer, W. E., *J. Biol. Chem.*, **165**, 393 (1946).
8. Patterson, J. M., Keevil, N. B., and McHenry, E. W., *J. Biol. Chem.*, **153**, 489 (1944). Friedlander, H. D., Chaikoff, I. L., and Entenman, C., *J. Biol. Chem.*, **158**, 231 (1945).
9. Frazer, A. C., *Nature*, **157**, 414 (1946).
10. Augur, V., Rollman, H. S., and Deuel, H. J., Jr., *J. Nutr.*, **33**, 177 (1947).
11. Frazer, A. C., and Stewart, H. C., *J. Physiol.*, **95**, 21 P, 23 P (1939).
12. Cooper, R. R., and Lusk, H., *Am. J. Digest. Dis.*, **9**, 395 (1942).
13. Elkes, J. J., Frazer, A. C., and Stewart, H. C., *J. Physiol.*, **95**, 68 (1939).
14. Rubner, M., Die Gesetze des Energieverbrauchs bei der Ernährung, Leipzig and Vienna, 281 (1902).
15. Gage, S. H., and Fish, P. A., *Am. J. Anat.*, **34**, 1 (1924).
16. Frazer, A. C., *J. Physiol.*, **102**, 329 (1943).
17. Boyd, D. R., and Marle, E. R., *J. Chem. Soc.*, **95**, 1807 (1910).
18. Fisher, R. A., Statistical methods for research workers, Edinburgh, 7th edition (1938).
19. Deuel, H. J., Jr., Hallman, L., and Leonard, A., *J. Nutr.*, **20**, 215 (1940).
20. André, E., and Bloch, A., *Bull. Soc. chim.*, **2**, 789 (1935).

THE THIOBARBITURIC ACID TEST APPLIED TO TISSUES FROM RATS TREATED IN VARIOUS WAYS

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When thiobarbituric acid (TBA) is heated with oxidized linolenic acid, a characteristic red color is produced. This color is proportional to the amount of linolenic acid oxidized, presumably to a peroxide, by autooxidation or in the presence of ascorbic acid or sulfhydryl compounds (1). Under the same conditions linoleic acid gives only traces of color which may result from the presence of small amounts of linolenic acid. Arachidonic, oleic, and the saturated fatty acids do not react, but a crude mixture of C_{20} acids does give the test. The TBA reagent can therefore be used to indicate the amount of oxidized linolenic acid, free or as phospholipide (2), in tissues, and possibly certain still unidentified C_{20} acids as well. The oxidation of linolenic acid which occurs when tissue slices or suspensions are incubated aerobically is primarily catalyzed by the ascorbic acid present (2). (Catalysis by sulfhydryl compounds occurs only below pH 4.0, and thus they would not be active at physiological hydrogen ion concentrations.) Incubation of tissues from scorbutic guinea pigs produces much less color (3), which is further evidence of the importance of ascorbic acid in the reaction.

In order to define further the usefulness of the test, rats were treated in several ways known to affect fat metabolism or fat deposition in various organs, and the tissues were subsequently analyzed. A group of animals was fasted 3 to 4 days. This is known to increase the fat content of the liver at the expense of the depots (4). Another group was given adrenalin a short time before being killed. Adrenalin is also known to increase the fat content of the liver (5-8) and to decrease the blood lipides (6, 7, 9). A third group was placed on the special fat-deficient diet described by Burr and Burr (10) and killed after the characteristic symptoms had appeared, usually after 3 to 4 months. Finally, two drugs which affect metabolism were tried. One group of rats was given 2,4-dinitrophenol for a week before their tissues were analyzed, and another was fed butter yellow on a high fat, low protein, low riboflavin diet for 6 months and then given a normal diet for 6 weeks before being killed. The results of the procedures are given below.

EXPERIMENTAL

The liver, kidney, and brain were analyzed. 1.0 gm. of tissue (wet weight) was ground in 5.0 ml. of 0.05 M phosphate buffer, pH 6.0. The suspension was strained through muslin. 0.1 ml. of it was added to 3.9 ml. of the buffer solution in a 50 ml. Erlenmeyer flask and incubated in air for 3 hours at 37°. At the end of the incubation, 1.0 ml. of 25 per cent trichloroacetic acid was added and the precipitated proteins were removed by centrifugation. To a 4.0 ml. aliquot of the supernatant 2.0 ml. of 1 per cent TBA and 4.0 ml. of H₂O were added. The mixture was put in a boiling water bath for exactly 5 minutes and the color read with a No. 540 filter in the Evelyn colorimeter. Tissue and reagent blanks were run as well as a calibration curve for the color produced per unit of linolenic acid, oxidized by aerobic incubation. The values thus obtained showed that the color was proportional to the amount of linolenic acid in low concentration ranges. No attempt was made to convert the color density into absolute amounts of linolenic acid, since it is not certain whether the oxidation in the presence and absence of tissue proceeds to the same endpoint. The finding of Bloor and Snider (11) that phospholipides after autoxidation become completely water-soluble accounts for the fact that the chromogen is found in the trichloroacetic acid solution. In order to determine whether any of it is precipitated with the protein, the TBA was added and the mixture boiled before the proteins were centrifuged. The values thus obtained agreed with those given by the former method. The test therefore indicates the amount of linolenic acid oxidized by the tissues under these experimental conditions. Addition of 0.2 mg. of ascorbic acid increased the values in all cases and gave an indication of the maximal amount of linolenic acid available for oxidation by this means. This value, which was not increased by either more ascorbic acid or longer incubation, was more significant than the one obtained without addition of ascorbic acid.

Table I summarizes the results. In all cases the addition of ascorbic acid to the tissue before incubation greatly increased the production of the chromogen. Of the three organs tested, the liver is the one primarily concerned with fat metabolism, and the values obtained under the different conditions showed the most striking changes. Fasting and adrenalin produced highly significant increases, both with and without ascorbic acid, whereas a fat-deficient diet produced a highly significant decrease, but only after addition of ascorbic acid. Dinitrophenol had no effect. Hepatomas from rats fed butter yellow gave low values, but since the range of values for the remaining tissue of normal appearance was wide the decrease is not significant. (In this connection it may be mentioned

that three spontaneous and three methyleholanthrene-induced rat tumors also gave very low values.) The same results are seen in the kidney, except that they were manifested primarily when the tissue was incubated with ascorbic acid. In the brain, only adrenalin caused an increase; fasting and fat deficiency had little effect. This was to be expected, since it has been shown that the brain maintains its normal fat content and presumably its normal fat metabolism under these conditions. The effect of adrenalin occurred rapidly and could be seen 10 minutes after intraperi-

TABLE I

TBA Test on Tissues from Rats Treated in Various Ways

The values are a measure of the color density (read in the Evelyn colorimeter) produced by 1 gm. of tissue (wet weight). The data represent the mean with standard deviations. The figures in parentheses represent the number of rats analyzed.

Condition	Brain	Brain + ascorbic acid	Kidney	Kidney + ascorbic acid	Liver	Liver + ascorbic acid
Normal.....	7.16 ±1.65 (10)	21.34 ±4.76 (7)	2.89 ±1.06 (10)	15.63 ±2.57 (7)	9.22 ±2.07 (10)	22.31 ±4.02 (7)
Fasting.....	8.58 ±1.72 (11)	27.03 ±3.98 (6)	3.98 ±1.39 (11)	20.05 ±2.59 (6)	14.41 ±4.39 (10)	39.73 ±5.19 (6)
Adrenalin..	11.62 ±2.23 (6)	38.18 ±6.39 (6)	3.70 ±1.06 (6)	19.77 ±5.19 (6)	15.48 ±4.56 (6)	39.69 ±10.52 (6)
Dinitro- phenol....	7.06 ±2.23 (9)	24.60 ±1.45 (4)	1.91 ±1.86 (9)	13.63 ±1.42 (4)	7.96 ±4.48 (9)	26.32 ±4.41 (4)
Fat-defi- cient.....	8.28 ±0.82 (10)	20.46 ±2.75 (10)	2.74 ±0.88 (10)	10.79 ±3.70 (10)	7.28 ±1.80 (12)	14.69 ±3.56 (12)
Butter yel- low.....	7.17 ±1.03 (7)	17.03 ±4.38 (7)	2.60 ±1.45 (7)	8.40 ±6.49 (7)	5.01 ±4.43* 3.33 ±2.96† (7)	13.97 ±8.64* 7.67 ±7.86† (7)

* Normal tissue.

† Tumor.

toneal injection of 7 mg. per kilo. It should be emphasized that *in vitro* adrenalin inhibits the oxidation of linolenic acid by ascorbic acid (2), and, if this happens at all *in vivo*, a decrease rather than an increase in values would occur.

Incubation of blood did not cause any increase in values under conditions which were optimal for the other tissues. A positive test can be obtained on whole blood if the TBA is heated with the proteins before they are centrifuged. 0.1 ml. of whole blood was heated for 5 minutes with 8.0

ml. of 10 per cent trichloroacetic acid and 2.0 ml. of TBA. The proteins were then removed by centrifugation and the color was read. Suitable blanks were run. The chromogen came primarily from the cells. Plasma alone gave a very faint test. The color densities thus obtained on three rats were 1.31, 1.09, and 0.90. Blood taken 2 hours after injection of adrenalin into the same animals gave 0.85, 0.72, and 0.72 respectively.

Table II presents the ratio of the values obtained with ascorbic acid divided by those obtained without its addition. It will be seen that ascorbic acid had more effect on the kidney than on brain or liver. This may be because the spontaneous oxidation in the kidney was small. The ratio is low in animals on the special fat-deficient diet, since in this condition linolenic acid is presumably present in concentrations lower than normal; thus ascorbic acid would have less effect. With dinitrophenol

TABLE II
Relative Effect of Ascorbic Acid on Various Tissues

The ratio equals the values obtained by incubating tissue with ascorbic acid divided by the values obtained without its addition.

Condition	Brain	Kidney	Liver
Normal.....	3.0	5.4	2.4
Fasting.....	3.1	5.1	2.8
Adrenalin.....	3.3	5.3	2.6
Dinitrophenol.....	3.5	7.1	3.3
Fat-deficient.....	2.6	4.3	1.9
Butter yellow.....	2.4	3.2	1.9
			2.7*

* Tumor.

the reverse is true; namely, the quotient increases, probably because less ascorbic acid is present. Svirebely (12) has, in fact, shown that dinitrophenol depletes the ascorbic acid content of tissues. The ratio is also low in the animals fed butter yellow, but the reason for this is not apparent.

The evidence indicates (2) that the oxidation product of linolenic acid may give rise to aldehyde groups, with possible breaking of the linkage at the double bond. It is not likely that the peroxide is reduced by any tissue component. After short aerobic incubation to produce the peroxide, tissues were incubated anaerobically or the product was added anaerobically to fresh liver, kidney, or brain. Incubation for 60 to 90 minutes did not reduce the amount of peroxide added at the beginning. The oxidation is, therefore, apparently irreversible in tissues, although it should be pointed out that Bloor and Snider (11) were able to oxidize leucomethylene blue by oxidized phospholipides.

DISCUSSION

The amount of oxidized linolenic acid, either free or combined, in the phospholipide molecule is measured by the TBA test. The oxidation product is in all probability a peroxide. Ascorbic acid is an excellent catalyst for this oxidation. It is possible to say, therefore, that by incubating tissues with an excess of ascorbic acid a value is obtained by the TBA test which denotes the amount of linolenic acid, and possibly certain C₂₀ acids, oxidizable by ascorbic acid. The results reported here, obtained by using procedures known to alter the fat distribution in various organs and presumably, therefore, their linolenic acid content, show that values obtained by the thiobarbituric acid test parallel those by the more conventional methods.

SUMMARY

1. The thiobarbituric acid test for the estimation primarily of the amount of oxidized linolenic acid present in tissues after aerobic incubation *in vitro*, with and without added ascorbic acid, was applied to the tissues of rats treated in ways known to alter the fat distribution.

2. The results obtained with this test parallel those obtained by the usual procedures for fat estimation.

3. In addition, it was shown that hepatomas produced by butter yellow give low values and that pretreatment with 2,4-dinitrophenol is without effect on the amount of oxidized linolenic acid estimated by the reagent under the experimental conditions.

BIBLIOGRAPHY

1. Wilbur, K. M., Bernheim, F., and Shapiro, O. W., *Arch. Biochem.*, in press.
2. Bernheim, F., Wilbur, K. M., and Fitzgerald, D. B., *J. Gen. Physiol.*, **31**, 195 (1947).
3. Abramson, H., *J. Biol. Chem.*, **178**, 179 (1949).
4. Dible, J. H., *J. Path. and Bact.*, **35**, 451 (1932).
5. Low, A., and Pfeiler, R., *Biochem. Z.*, **193**, 277 (1928).
6. Page, I. H., and Pasternak, L., *Biochem. Z.*, **232**, 295 (1931).
7. Pollack, A., *Compt. rend. Soc. biol.*, **128**, 76 (1938); **130**, 149 (1939).
8. Sveinsson, S. L., *Skand. Arch. Physiol.*, **77**, 79 (1937).
9. Fleisch, A., *Biochem. Z.*, **177**, 460 (1926).
10. Burr, G. O., and Burr, M. M., *J. Biol. Chem.*, **82**, 345 (1929); **86**, 587 (1930).
Burr, G. O., Burr, M. M., and Miller, E. S., *J. Biol. Chem.*, **97**, 1 (1932).
11. Bloor, W. R., and Snider, R. H., *Proc. Soc. Exp. Biol. and Med.*, **36**, 215 (1937).
12. Svrbely, J. L., *Am. J. Physiol.*, **116**, 446 (1936).

DISPLACEMENT ANALYSIS OF LIPIDES

III. SEPARATION OF NORMAL SATURATED FATTY ACIDS FROM FORMIC TO BEHENIC*

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Displacement separation is a special technique of chromatographic separation developed by Tiselius (1), in which the column is developed by a solution of a substance which is adsorbed more strongly than the components of the sample. After the column is washed, the sample in a minimal volume of solvent is pressed into the column. A solution of the displacer is then pressed into the column, and because it is more strongly adsorbed than are the components of the sample, they are displaced and migrate down the column ahead of the displacer, forming zones in the order of their adsorbabilities, the least adsorbed substance migrating first. Each substance in the chromatogram thus displaces the component with the next lower adsorbability and the components migrate in zones one after the other. The zones are detected and the concentrations of solute in the effluent are measured interferometrically.

This type of separation has been applied to the group separation of branched, unsaturated, and normal saturated acids by Claesson (2), but he was unable to achieve displacement separation of homologous fatty acids (3). The possibility of separation of the members of the saturated normal series by displacement was indicated by the preliminary experiments of the present authors (4) in which they employed the coupled filters of Hagdahl (5). Recently Hagdahl and Holman studied the effect of solvent upon the apparent separation of homologous fatty acids and found that separation was enhanced by use of a solvent in which the displacer is in nearly saturated solution (6). Application of this principle in the choice of solvent made it possible to demonstrate separation of all the normal saturated fatty acids from C_1 to C_{22} with the exception of the C_{21} acid. Separation of homologues differing by 1 carbon atom has been demon-

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strated by the use of five displacement set-ups with from three to six acids per set-up.

EXPERIMENTAL

All experiments were performed with a modification of the Tiselius-Claesson interferometric adsorption analysis apparatus (7), equipped with a 76 mm. cuvette and the coupled filters and mixer of Hagdahl (5). All experiments were performed with a mixture of 1 part of Darco G-60 and 2 parts of Hyflo Super-Cel as adsorbent. The columns were driven with a pressure of approximately 40 pounds per sq. in. with a speed of about 10 to 20 ml. per hour.

The docosanoic acid, m.p. 78° , was prepared by hydrogenation of erucic acid which was isolated from rape seed oil. Eicosanoic acid, m.p. 72° , and heptadecanoic acid, m.p. 57° , were supplied by Einar and Stina Stenhagen. Pentadecanoic acid, m.p. 52° , was obtained from Professor Schuette. Undecanoic acid, m.p. 26° , was prepared by hydrogenation of undecylenic acid. All other fatty acids were Eastman products. Displacement or frontal analyses of the single fatty acids showed no significant impurities in butyric, valeric, octanoic, decanoic, dodecanoic, tetradecanoic, hexadecanoic, octadecanoic, and docosanoic acids. Hexanoic, heptanoic, nonanoic, and tridecanoic acids were found to be inhomogeneous by single displacement experiments, but the impurities apparently were higher or lower homologues, for their presence did not complicate the displacement diagrams in which these acids were separated from their homologues, except in the case of hexanoic acid. Single displacement diagrams were not made for undecanoic, pentadecanoic, heptadecanoic, nonadecanoic, and eicosanoic acids because of the limited amounts of these acids available.

In choosing experimental conditions, solvent mixtures were sought in which the desired concentration of displacer fatty acid was close to the limit of solubility. This solvent mixture was then used to wash the column prior to use, as solvent for the sample and as solvent for the displacer. Usually with mixed solvents an artifact was observed in the displacement diagram in the form of a step just prior to the first emerging acid. This artifact is probably a displaced solvent emerging in a concentration different from that in which it was introduced. The composition of the mixed solvents is expressed as percentage by volume.

RESULTS AND DISCUSSION

For the separation of the lower homologues of the fatty acid series, water was chosen as solvent, for although the lower acids are very soluble in water, the acids of medium chain length are of limited solubility. From Fig. 1 it will be seen that formic and acetic acids emerged as elution peaks

rather than as displacement steps. However, beginning with propionic acid, the higher homologues established themselves in equilibrium concentrations with the charcoal and emerged as well defined displacement steps. If a solvent were used in which formic and acetic acids are of lower solubility, it seems likely that they too could be separated into displaced zones on the column rather than as elution peaks. The extra step appearing ahead of the hexanoic acid zone is an impurity associated with the hexanoic acid. This impurity has appeared in all displacement and frontal analyses in which this sample of hexanoic acid has been used.

In the series of acids with from 6 to 12 carbon atoms it was necessary to choose a higher displacer concentration, for in the 50 per cent alcohol the refractive index increment for the fatty acid concentration normally used

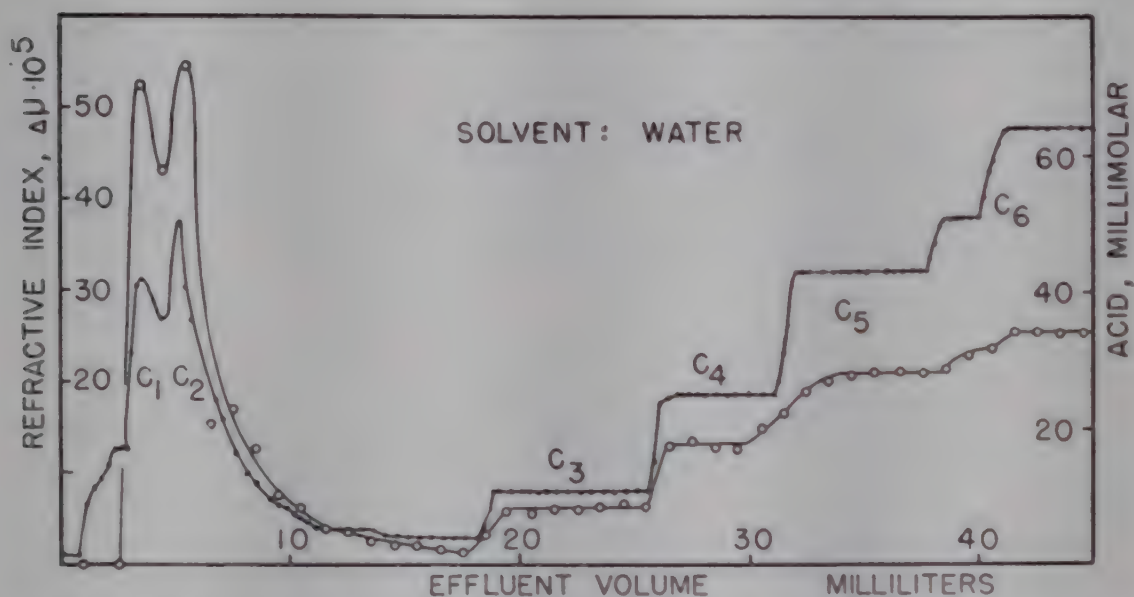


FIG. 1. Displacement diagram for acids with 1 to 6 carbon atoms. Sample, 10 mg. of formic, 10 mg. of acetic, 5 mg. of propionic, 10 mg. of butyric, and 20 mg. of valeric acids. Solvent, water. Filter, $800 + 400 + 200 \text{ } \mu \text{ c.m.m.} = 4.4 \text{ ml.}$ Displacer, 0.4 per cent hexanoic acid. \circ acid by titration; \bullet refractive index.

(1 per cent) was low, thereby making recognition of the steps more difficult. With dodecanoic (lauric) acid as displacer, the separation of decanoic, nonanoic, octanoic, heptanoic, and hexanoic acids is readily apparent in the displacement diagram shown in Fig. 2.

In Fig. 3 the separation of decanoic, undecanoic, dodecanoic, and tridecanoic acids is shown with tetradecanoic (myristic) acid as displacer. For this displacement separation 65 per cent alcohol was found suitable. The separation of tetradecanoic, pentadecanoic, and hexadecanoic acids in 80 per cent alcohol is shown in Fig. 4.

To demonstrate the separability of the acids with 16 to 20 carbons it was necessary to employ as displacer docosanoic (behenic) acid, the only readily available saturated fatty acid with more than 20 carbon atoms. The very

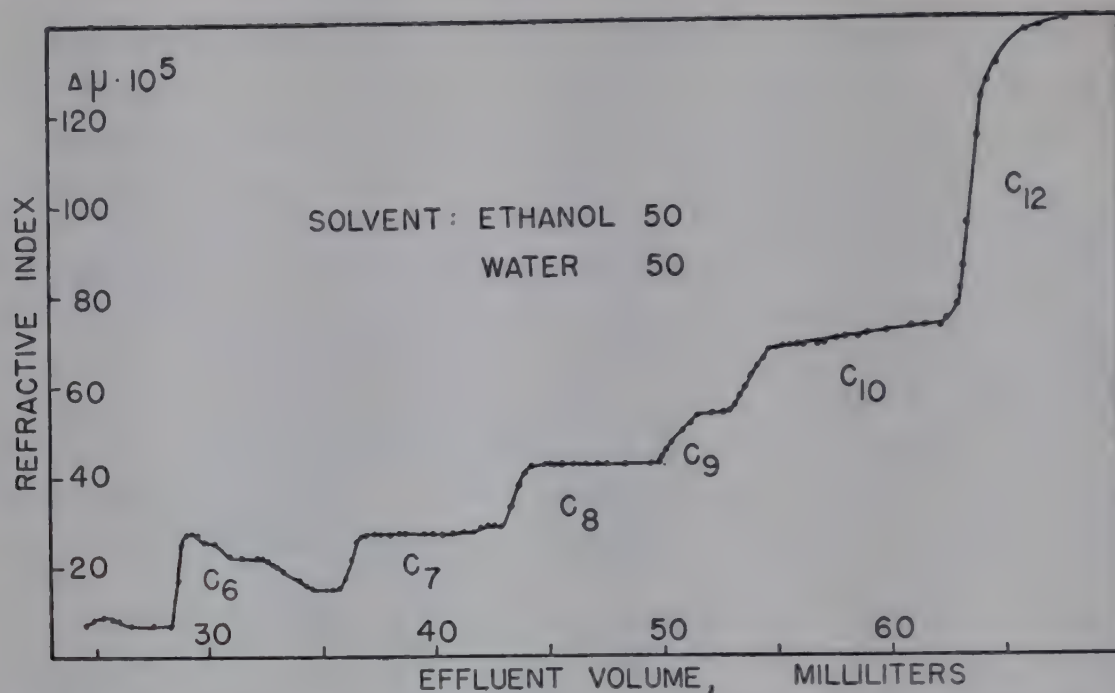


FIG. 2. Displacement diagram of acids with 6 to 12 carbon atoms. Sample, 25 mg. of hexanoic, 40 mg. of heptanoic, 60 mg. of octanoic, 80 mg. of nonanoic, and 140 mg. of decanoic acids. Solvent, 50 per cent alcohol. Filter, 5000, 2000, 800, 400, 200, 100 μ c.mm. = 26.7. ml. Displacer, 2.0 per cent dodecanoic acid.

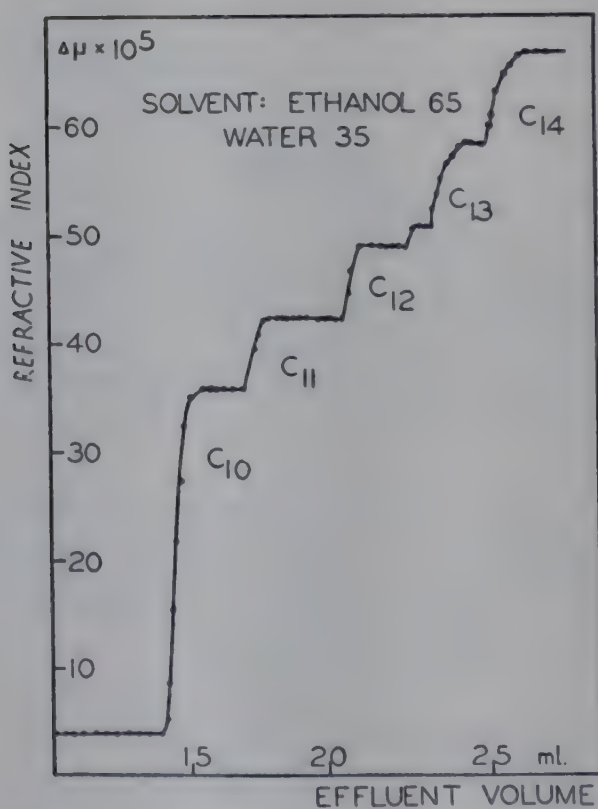


FIG. 3. Displacement diagram for acids of 10 to 14 carbon atoms. Sample, 20 mg. of decanoic, 30 mg. of undecanoic, 40 mg. of dodecanoic, and 50 mg. of tridecanoic acids. Solvent, 65 per cent alcohol. Filter, 2000, 800, 400, 100 μ c.mm. = 10.4 ml. Displacer, 1.0 per cent tetradecanoic acid.

low solubility of this acid in alcohol limits its use as a displacer in alcohol. However, by the use of 22 volumes per cent chloroform in the alcohol, the solubility of docosanoic acid can be raised above 1.0 per cent. With 1.0 per cent docosanoic acid in this solvent, the displacement separation of hexadecanoic, heptadecanoic, octadecanoic, nonadecanoic, and eicosanoic acids is demonstrated in Fig. 5. Unfortunately the acid with 21 carbon atoms was not available for use, but undoubtedly it too could be separated from its nearest homologues by this method.

Fatty acids have been separated by other chromatographic techniques.

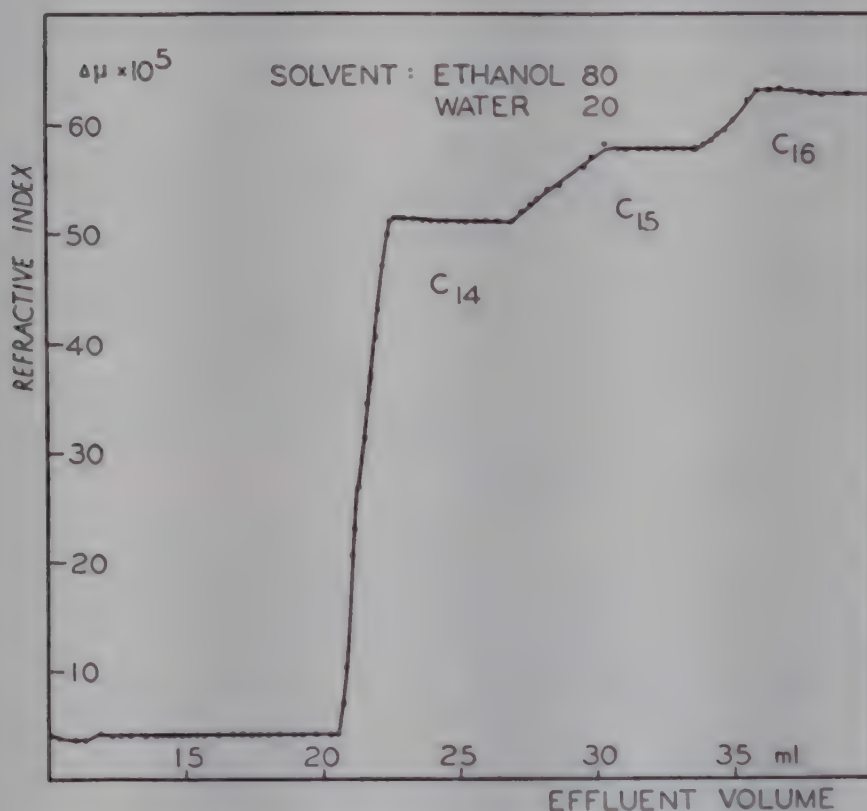


FIG. 4. Displacement diagram for acids of 14 to 16 carbon atoms. Sample, 45 mg. of tetradecanoic and 60 mg. of pentadecanoic acids. Solvent, 80 per cent alcohol. Filter, 2000, 800, 400, 200, 100 μ c.mm. = 11.0 ml. Displacer, 1.0 per cent hexadecanoic acid.

Smith (8) found that silica gel partition chromatography could be used to separate formic, acetic, propionic, butyric, and valeric acids. Ramsey and Patterson (9) also were able to separate formic through butyric acids by partition chromatography. Peterson and Johnson (10) and Ramsey and Patterson (11) have extended the usefulness of partition chromatography to allow separation of fatty acids as high as 10 carbon atoms in length. However, acids above 10 carbon atoms in length have not been separated by this method, because their partition coefficients become nearly identical.

Elution chromatography has been used by several investigators (12-16)

for the separation of fatty acids, but this technique has the disadvantage that yields often are low and that great volumes of liquid, and consequently long periods of time, are required to develop the chromatogram. Displacement separation circumvents this objection, for the development of chromatograms such as are presented here requires only a few hours with a minimal volume of effluent. From the results presented here it is clear that the displacement technique can be used for the whole range of commonly occurring saturated fatty acids. It is reasonable to assume that its usefulness can be extended even farther up the fatty acid series by altering the solvent to increase the solubility of the higher fatty acids. In this

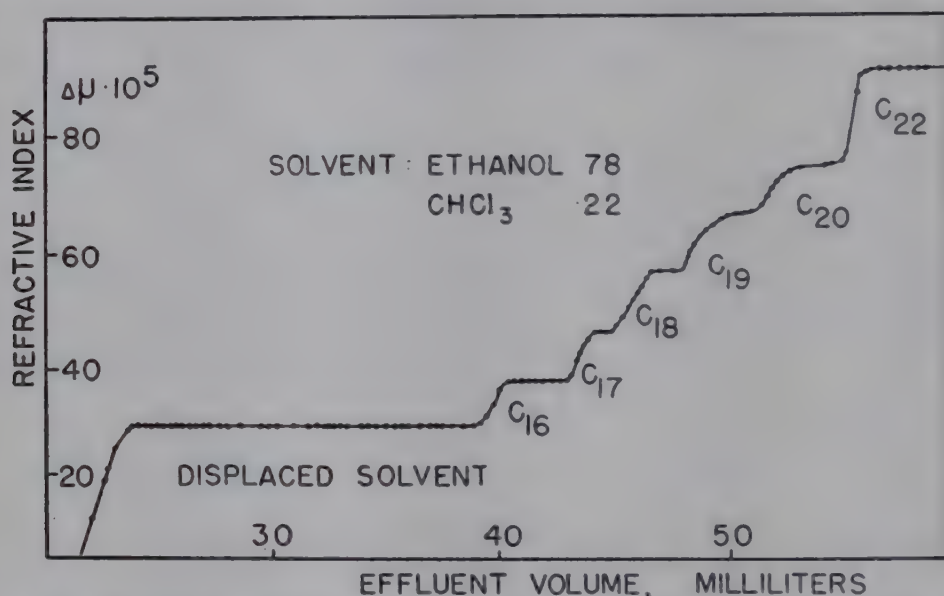


FIG. 5. Displacement diagram for acids of 16 to 22 carbon atoms. Sample, 12 mg. of hexadecanoic, 15 mg. of heptadecanoic, 20 mg. of octadecanoic, 30 mg. of nonadecanoic, and 35 mg. of eicosanoic acids. Solvent, chloroform 22 and ethanol 78 volumes per cent. Filter, 2000, 800, 400, 200, 100, 50 μ c.mm. = 11.2 ml. Displacer, 1.0 per cent docosanoic acid.

regard, the difficulty now seems to be to find a readily available displacer with a longer carbon chain.

If the technique of displacement were to be used in studies of naturally occurring saturated fatty acids, even better separations and more clearly discernible steps could be expected, for the even numbered carbon chain acids predominate in natural lipides. In the common fats and oils odd numbered acids are absent, and separations would involve only acids differing by 2 carbon atoms. However, unsaturated acids may possibly complicate separations and require close observation because of only slight differences in refractive index between zones (step height). In a few preliminary separations involving unsaturated fatty acids this has not been a source of trouble, and it appears that the displacement technique will be applicable to mixtures of unsaturated and saturated fatty materials (17).

The studies here presented indicate the possibility of separation of homologous fatty acids with as little difference as 1 carbon atom over the entire range of chain lengths usually encountered in common fats. No attempts to follow yields have been made in the present study, but yields of about 95 per cent have been found in earlier experiments (5). It is intended that additional studies be made to adapt displacement separation to the quantitative analysis of fatty acids.

SUMMARY

1. Displacement separation of fatty acids has been shown to take place for all normal saturated acids of from 1 to 20 carbon atoms in length by proper choice of solvent for each group of acids, with Darco G-60 as adsorbent.

2. Formic through hexanoic acids were separated with water as solvent. Hexanoic through dodecanoic acids were separated in 50 per cent alcohol. Decanoic through tetradecanoic acids were separated in 65 per cent alcohol. Separation of tetradecanoic, pentadecanoic, and hexadecanoic acids was accomplished in 80 per cent alcohol. For the separation of hexadecanoic through eicosanoic acids 22 per cent chloroform in alcohol was used as solvent and docosanoic acid as displacer.

BIBLIOGRAPHY

1. Tiselius, A., *Ark. Kemi, Mineral. o. Geol.*, **16 A**, 18 (1943).
2. Claesson, S., *Rec. trav. chim. Pays-bas*, **T65**, 571 (1946).
3. Claesson, S., *Ark. Kemi., Mineral. o. Geol.*, **23 A**, 1 (1946).
4. Holman, R. T., and Hagdahl, L., *Arch. Biochem.*, **17**, 301 (1948).
5. Hagdahl, L., *Acta chem. Scand.*, **2**, 574 (1948).
6. Hagdahl, L., and Holman, R. T., *J. Am. Chem. Soc.*, in press.
7. Tiselius, A., and Claesson, S., *Ark. Kemi., Mineral. o. Geol.*, **15 B**, 18 (1942).
8. Smith, E. L., *Biochem. J.*, **36**, p. xxii (1942).
9. Ramsey, L. L., and Patterson, W. I., *J. Assn. Official Agr. Chem.*, **28**, 644 (1945).
10. Peterson, M. H., and Johnson, M. J., *J. Biol. Chem.*, **174**, 775 (1948).
11. Ramsey, L. L., and Patterson, W. I., *J. Assn. Official Agr. Chem.*, **31**, 139 (1948).
12. Kaufman, H. P., and Wolf, W., *Fette u. Seifen*, **50**, 519 (1943).
13. Cassidy, H. G., and Wood, S. E., *J. Am. Chem. Soc.*, **63**, 2628 (1941). Cassidy, H. G., **63**, 2735 (1941).
14. Dutton, H. J., *J. Phys. Chem.*, **48**, 179 (1944).
15. White, M. F., and Brown, J. B., *J. Am. Chem. Soc.*, **70**, 4269 (1948).
16. Riemenschneider, R. W., Herb, S. F., and Nichols, P. L., *J. Am. Oil Chem. Soc.*, **26**, 371 (1949).
17. Holman, R. T., and Hagdahl, L., Abstracts, American Chemical Society, 115th meeting, San Francisco, 5C (1949).

PAPER CHROMATOGRAPHIC SEPARATION AND ULTRA-VIOLET ANALYSIS OF COMMERCIALY PREPARED PROGESTERONE

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The applications of paper chromatography to the separation and subsequent analysis of various substances are rivaled only by the number of substances to be separated. The theoretical and practical aspects of this relatively new concept (1) have been discussed in detail in a recent review of the subject (2).

The assay of commercially prepared progesterone in oily vehicles such as sesame or cottonseed oil is rendered exceedingly difficult by conventional chemical separatory maneuvers because of the relatively similar physical properties of progesterone and the oils in which it is supplied. Bioassay is also difficult and tedious because of the large number of test animals that must be used to render a given assay statistically significant. It was, therefore, decided to attempt a separation of progesterone from its vehicle by paper chromatography by using an immiscible biphasic solvent system of butanol and water. Preliminary experiments concerned with this mode of separation failed to yield the expected results in that a large percentage of the oil and the progesterone flowed over the paper, and an adequate separation did not occur. The next step was to use another solvent system. Since progesterone is soluble in 80 per cent ethanol and sesame and cottonseed oil are not, a unisolvant system with 80 per cent ethanol was devised with the following results.

EXPERIMENTAL

A paper partition system was contrived in which only standard laboratory equipment was utilized. The outside container was made with a large desiccator bowl as the bottom and a tall bell jar, which fitted tightly upon the bowl, as the top. A high glass stand was introduced into this, the bottom of which rested in a container of 80 per cent ethanol, the latter used to insure atmospheric saturation. The holder of the paper strips and the reservoir for the 80 per cent ethanol at the top of the system were a glass slide container 10 × 8 × 8 cm. Whatman No. 1 filter paper was

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used throughout the experiment and was cut into strips 3×40 cm. Sesame and cottonseed oil samples were obtained from pharmaceutical manufacturers¹ and were subjected to paper chromatography. Standard solutions of progesterone in sesame and cotton oils were made in this laboratory for separation and analysis. Four "unknown" progesterones in oil samples were prepared by one of us for analysis. Since relatively minute amounts of oil solutions (0.005 to 0.1 cc.) were placed on the strips for analysis, the oil was diluted ten times with carbon tetrachloride to facilitate its application and also to preclude, as much as possible, errors in pipetting.

A measured amount of the solution to be chromatographed was spread evenly along a penciled starting line on the strip, 8 cm. from one end of the paper. The oil was then allowed to dry. The alcohol container at the bottom of the system was placed in its position in the desiccator bottom. The end of the strip nearest the oil was then placed in the top reservoir, the bell jar put in place, and the system closed for a period of about 16 to 18 hours. At the end of this time the strips were removed and allowed to dry at room temperature.

The next procedure was to locate the progesterone. This could be accomplished by sectioning the strip in 1 or 2 cm. segments, placing them in 95 per cent ethanol, and subjecting the resultant solution to ultraviolet analysis. The presence of absorption at $240\text{ m}\mu$ indicated the presence of progesterone. This proved to be tedious. Therefore, a chemical test for progesterone on paper was devised by using the knowledge that progesterone contains a ketone linkage and that *m*-dinitrobenzene, in the presence of alkali and keto groups, gives a color response. The strip was immersed in a saturated alcoholic solution of *m*-dinitrobenzene and was heated to dryness and then subjected to a 5 N aqueous solution of potassium hydroxide by immersion and drying. As the strip dried, the presence of progesterone was heralded by the production of a brilliant blue color. This modification of the Zimmermann test is not specific for progesterone and has been demonstrated to occur in the presence of similarly constructed molecules such as desoxycorticosterone and testosterone. The 17-keto steroids do not give a blue color on paper, but instead their presence can be determined by the occurrence of a violet color.

It was found that under controlled conditions the R_F of progesterone

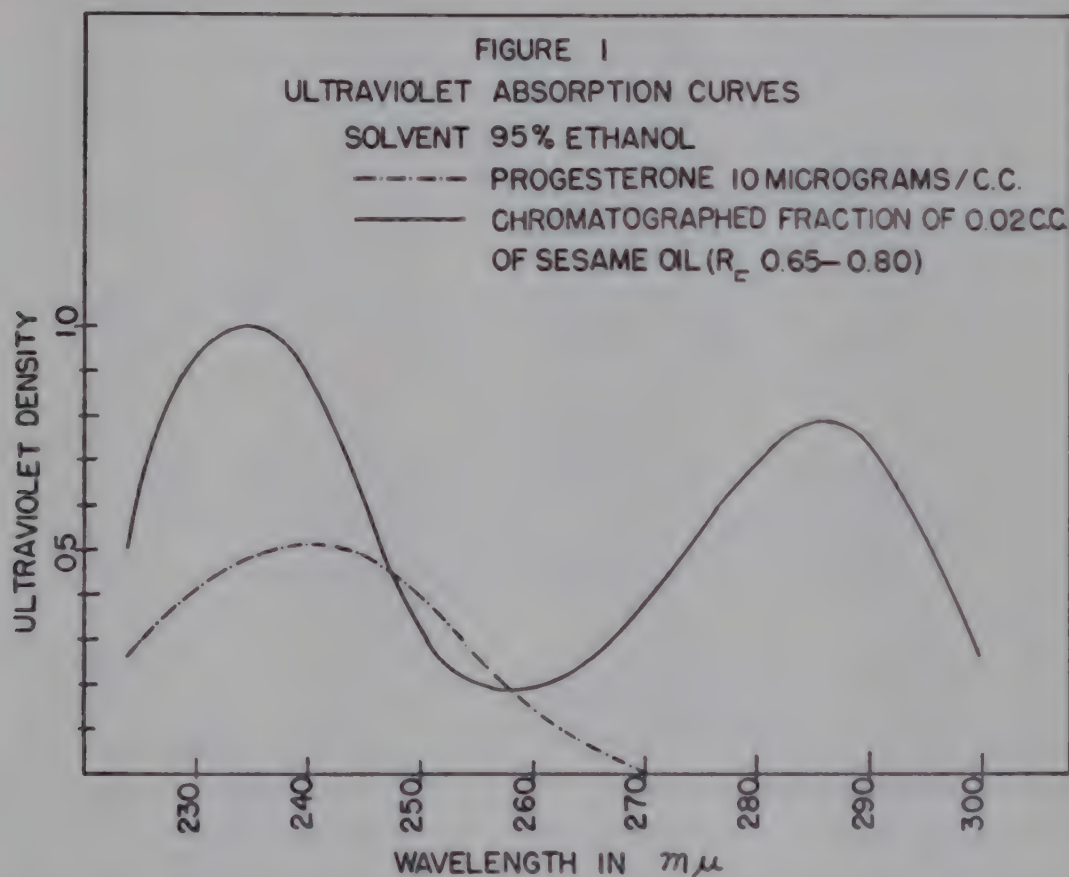
$$\frac{\text{Distance progesterone has traversed}}{\text{Distance fluid front has traversed}}$$

was fairly constant with a mean of 0.84. Each strip to be analyzed was sectioned lengthwise into a 1 cm. and a 2 cm. strip. The former was desig-

¹ Winthrop-Stearns, Inc., the Schering Corporation, The Upjohn Company, and Eli Lilly and Company contributed the oils for analysis.

nated as the indicator strip and the progesterone located with the *m*-dinitrobenzene reagent. The indicator strip was then compared to the untreated strip and by projection the location of the progesterone was approximated. The area containing the progesterone and the 2 cm. segments in bilateral juxtaposition was cut from the mother strip and each placed in 5 cc. of 95 per cent ethanol for ultraviolet analysis.

The Beckman spectrophotometer, with 1 cm. square fused silica absorption cells, was used in making the ultraviolet analyses. The absorption curves obtained were compared to a standard progesterone absorption



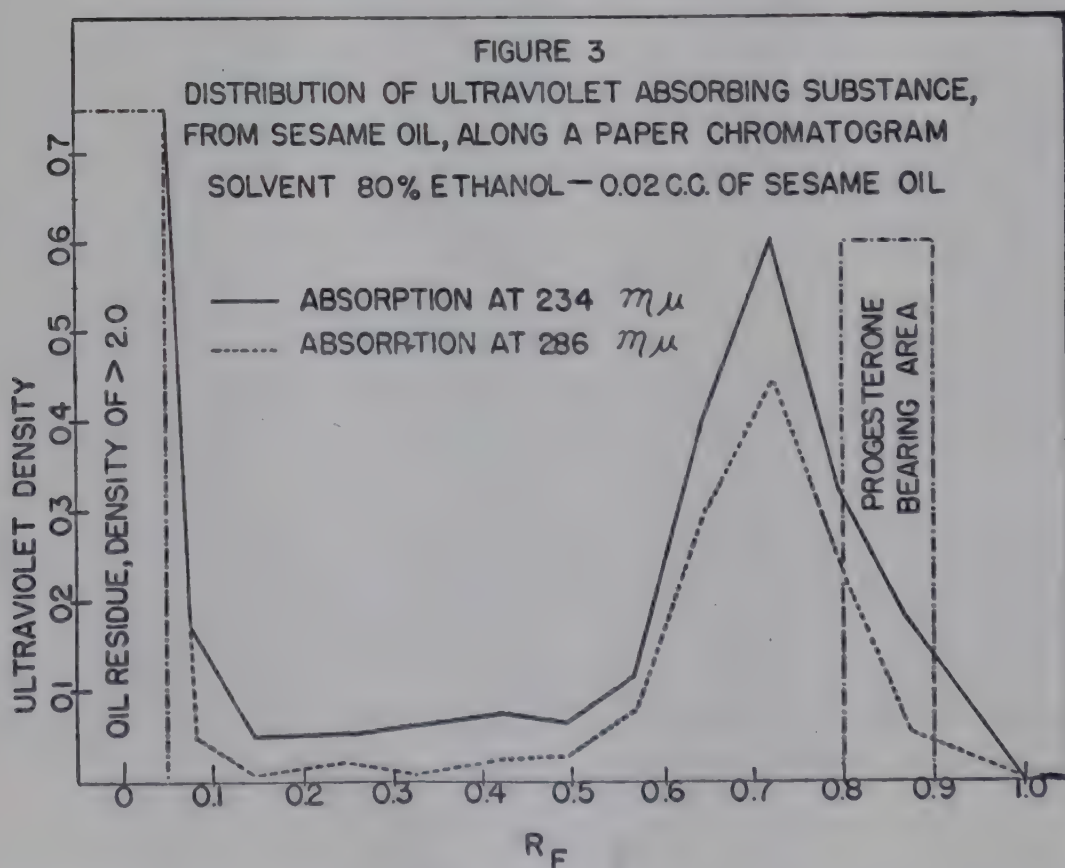
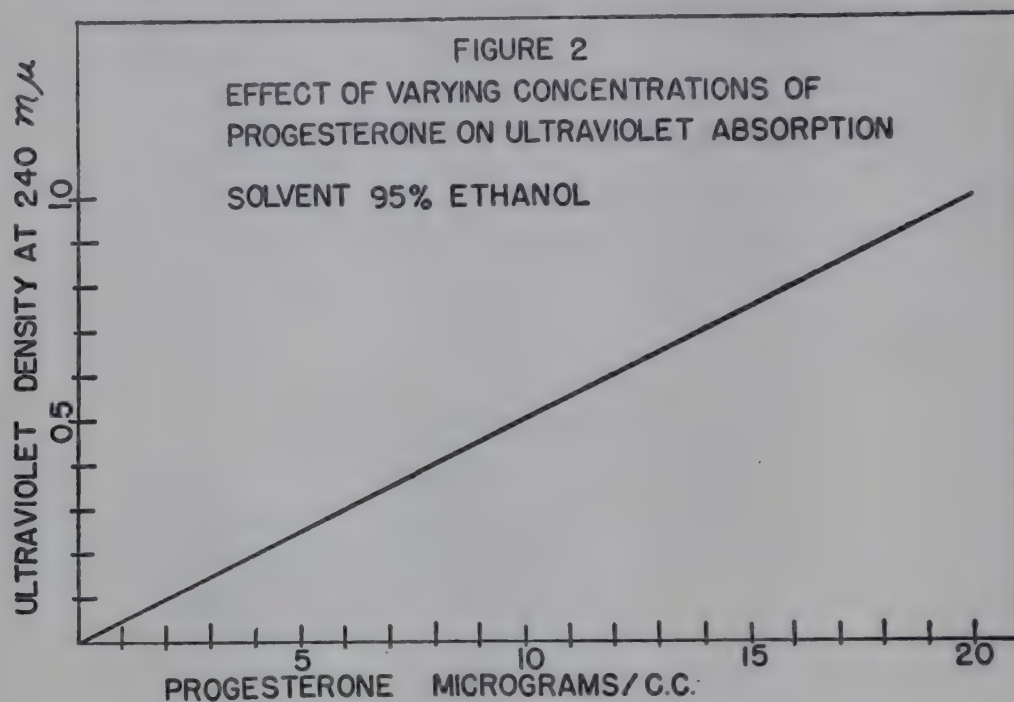
curve, as indicated in Fig. 1. Amounts of progesterone were calculated from the density at 240 $m\mu$, and the standard as graphed in Fig. 2.

The samples of cottonseed oil and sesame oil were chromatographed and analyzed by using ultraviolet absorption and the *m*-dinitrobenzene test to demonstrate that, in the absence of progesterone, there was neither absorption at 240 $m\mu$ nor the production of a blue color.

Results

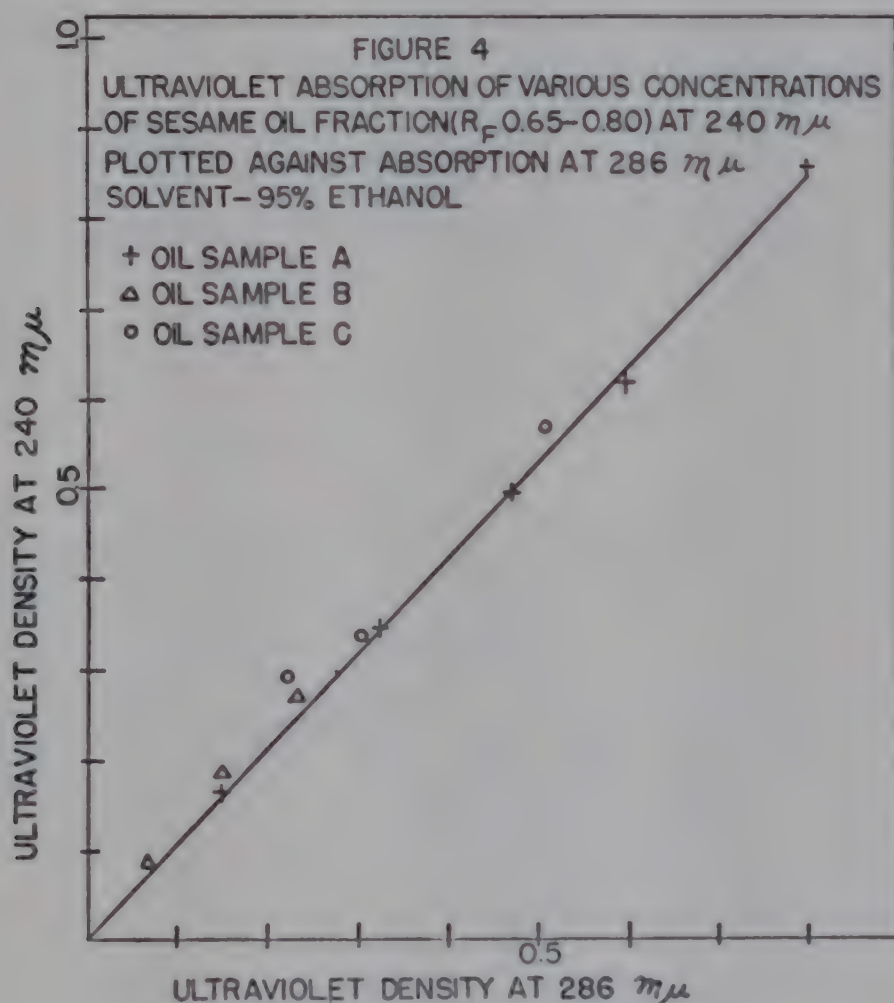
The three sesame oil samples studied yielded interesting results when analyzed. Since there was no apparent difference noted among them, only one is presented for discussion. There was no gross evidence of oil spread beyond the starting line. It will be noted in Fig. 3 that there is a

strongly ultraviolet absorptive substance present on the strip between the R_F of 0.65 and 0.80. The curve obtained was characteristically the same



in all sesame samples tested. The curve (Fig. 1) shows two absorption bands, the first with a peak at $234\text{ m}\mu$, and the second with its peak at $286\text{ m}\mu$. This substance at times interfered with the ultraviolet analysis of

progesterone when perfect separation was not achieved. The presence of the contaminating substance was always readily discovered by the secondary absorption band, since progesterone alone fails to show absorption at 286 $m\mu$. Accordingly the analyses which showed contaminating substances could be either discarded or corrected. It was considered to be more desirable to derive a correction factor. All that was required of a correction factor was one that could predict with accuracy the interfering density at 240 $m\mu$, that is density at 240 $m\mu$ not due to progesterone. An analysis of all the absorption curves obtained from the contaminating fraction of the

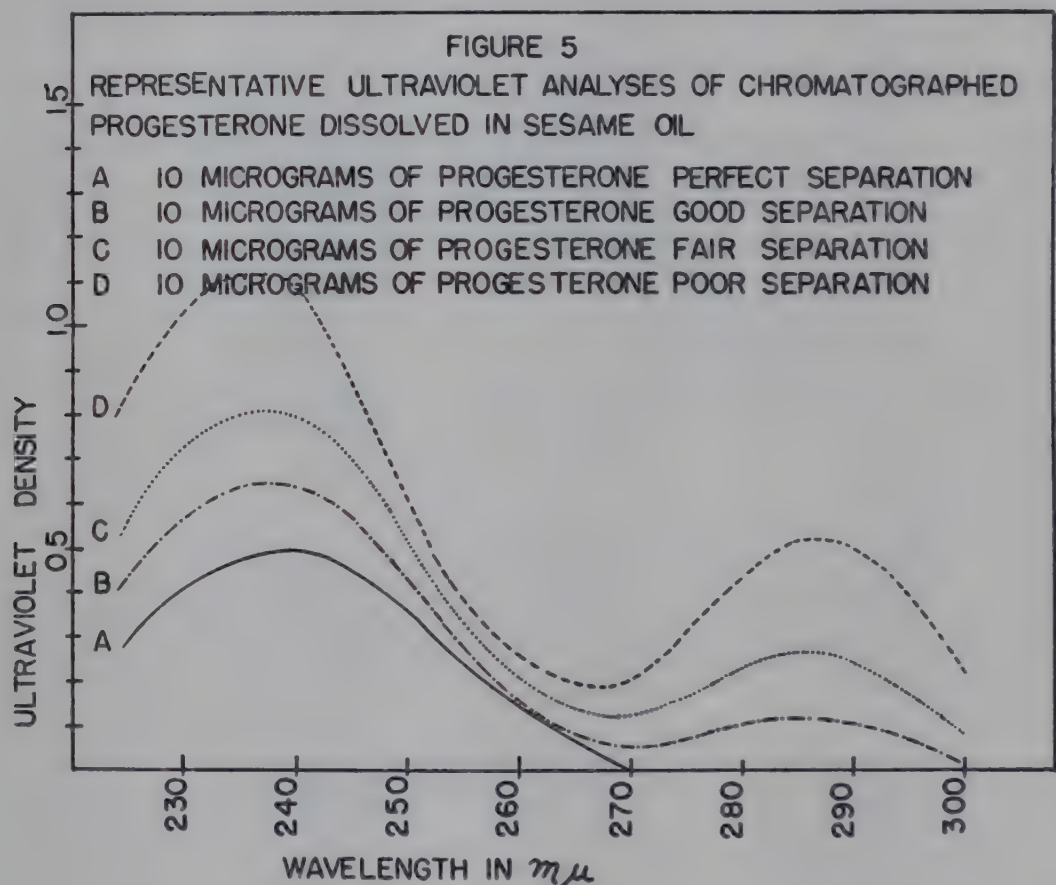


sesame oil, R_F 0.65 to 0.80, revealed many common characteristics, but the most applicable finding was that the density at 240 $m\mu$ varied directly with the observed density at 286 $m\mu$. This relationship was found to be constant in the three sesame oil samples and is graphed in Fig. 4. Accordingly contaminating density at 240 $m\mu$ can be calculated by the following:

$$D_{240} = \frac{OD_{286}}{0.944}$$

where D is the predicted density at $240\text{ m}\mu$ from the contaminating substance and OD is the observed density at $286\text{ m}\mu$.

Reference to Fig. 5 and calculation will further illustrate the practical aspect of this computation. Curve A represents the absorption pattern of $10\text{ }\gamma$ of progesterone, and shows a density of 0.500 at $240\text{ m}\mu$. Curve B shows the resultant curve of $10\text{ }\gamma$ of progesterone plus a small amount of contaminating substance, with densities of 0.640 at $240\text{ m}\mu$ and 0.135 at $286\text{ m}\mu$.



This is corrected as follows:

$$D_{240} = \frac{OD_{286}}{0.944}; D_{240} = \frac{0.135}{0.944}; D_{240} = 0.143$$

Subtraction of the predicted density at 240 of 0.143 (produced by the contaminating substance) from the total observed density at 240 of 0.640 then yields the density due only to the progesterone, in this case 0.497 . This figure compares favorably to the theoretical absorption of $10\text{ }\gamma$ of progesterone at $240\text{ m}\mu$ of 0.500 . A similar analysis of Curves C and D finds the density due to the $10\text{ }\gamma$ of progesterone to be 0.498 and 0.502 , respectively.

Analysis of the segment indicated to contain the progesterone always contained the major portion of this substance, but the occasional appear-

TABLE I

Chromatographic Separation and Ultraviolet Analysis of Known Concentrations of Progesterone in Sesame Oil

Expressed as mg. of progesterone per cc. of oil. The control was 0.000.

Assay No.	2 mg. per cc.	5 mg. per cc.	10 mg. per cc.	25 mg. per cc.
1	1.780	5.400	8.900	30.000
2	2.110	5.750	9.800	30.500
3	1.620	4.600	8.530	20.250
4	1.720	5.800	10.800	29.500
5	1.756	4.710	10.000	25.300
6	1.800	5.600	9.200	22.300
7	1.668	4.550	9.000	24.000
8	1.890	4.230	10.700	24.500
9	2.256	5.200	8.540	23.200
10	2.000	4.850	8.180	26.000
Mean recovery.....	1.860	5.069	9.365	25.615
" deviation.....	0.163	0.481	0.768	2.828
" recovery, %.....	93.0	101.4	93.7	102.5
" deviation, %.....	8.2	9.6	7.7	11.3

TABLE II

Chromatographic Separation and Ultraviolet Analysis of "Unknown" Concentrations of Progesterone in Sesame Oil

Expressed as mg. of progesterone per cc. of oil.

Assay No.	2 mg. per cc.	4 mg. per cc.	8 mg. per cc.	16 mg. per cc.
1	1.268	3.730	7.745	14.448
2	1.833	3.334	7.745	16.470
3	1.636	3.975	7.701	14.970
4	1.778	3.625	7.885	17.586
5	2.768	4.235	8.029	14.358
6	2.640	3.751	7.953	14.724
7	2.273	4.025	8.025	14.431
8	2.038	3.920	7.606	15.692
9	1.640	3.714	8.231	14.934
10	1.700	3.344	7.806	16.674
11	1.640	4.231	8.302	15.738
12	1.633	3.941	8.509	15.210
Mean recovery.....	1.904	3.819	7.961	15.436
" deviation.....	0.351	0.235	0.223	0.830
" recovery, %.....	95.2	95.5	99.5	96.5
" deviation, %.....	18.4	6.2	2.8	5.4

ance of the steroid in the contiguous segments necessitated their analysis. Table I is a compilation of 50 chromatographic separations and subsequent analysis with the resultant means and deviation. It is considered that ten runs on each sample to be tested yield an adequate analysis. Table II showed the result of forty-eight analyses of "unknown" amounts of progesterone in sesame oil. Following the analysis of these samples, the actual amount of progesterone placed in solution was disclosed. These results do not differ significantly from the "knowns." The analysis of the cottonseed oil with progesterone added yielded equally as satisfactory results as those separations in which sesame oil was used as the vehicle. Cottonseed oil varied from sesame oil, in that there was no highly absorptive material encountered on the strip; nor was there any evidence of the 234 $m\mu$ or 286 $m\mu$ absorption bands.

Trial runs of desoxycorticosterone acetate and testosterone propionate indicated that these substances may be separated with equal facility from sesame oil. Further, since they too have an absorption band similar to progesterone and since they react similarly to the *m*-dinitrobenzene and potassium hydroxide, this technique of analysis for progesterone is directly applicable.

DISCUSSION

It is obvious that this procedure actually does not identify the substance separated from the oil as progesterone but defines it only as a ketone by the *m*-dinitrobenzene reagent and an α,β -ketone because of the ultra-violet absorption at 240 $m\mu$. A confirmatory test should be made to determine the physiological activity of the substance. For this purpose we prefer the original bioassay method, as described for progesterone by Corner and Allen (3). It should be emphasized that it is necessary to test only one or two animals at a proper dosage level to determine biological activity, but no attempt should be made to give quantitative significance to the bioassay. The method used in this separation of progesterone from oil differs from the conventional paper chromatographic methods. Usually a biphasic solvent system is utilized in which the solvents are immiscible. Theoretically, the solvents spread on the paper in two layers and the resultant distribution of the separated substances is dependent upon their relative solubility in the solvents. The use of a monophasic solvent system is considered to be unique and may be of general use in securing adequate separations which are not suitable for conventional paper chromatography.

SUMMARY

Progesterone may be separated in a relatively pure state and in micro amounts from the oils in which it is commercially supplied, utilizing paper

chromatography. The progesterone is located on the paper strip by applying *m*-dinitrobenzene and potassium hydroxide. The progesterone on the strip is measured quantitatively, after it is dissolved in 95 per cent ethanol, by ultraviolet analysis. A factor is presented as a correction for the small amounts of interfering substance present.

BIBLIOGRAPHY

1. Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, **38**, 224 (1944).
2. Strain, H. H., *Anal. Chem.*, **21**, 75 (1949).
3. Corner, G. W., and Allen, W. M., *Am. J. Physiol.*, **88**, 326 (1929).

DIMORPHISM OF FORMYL-D- AND L-METHIONINE AND THE EFFECT OF HYDROCHLORIC ACID ON THE ROTATION OF D- AND L-METHIONINE

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This paper describes a case of dimorphism of formyl-D- and L-methionine and the effect of hydrochloric acid on the optical rotation of D- and L-methionine.

EXPERIMENTAL

DL-Methionine was resolved as described by Windus and Marvel (1) and by Jackson and Block (2). The latter authors observed that crystallization of the more soluble brucine formyl-L-methionine was prevented because of interference by excess brucine. To obviate this, they evaporated the ethanolic solution obtained after removal of brucine formyl-D-methionine to dryness and then separated the excess brucine from the brucine formyl-L-methionine by the difference of their solubilities in water. A similar procedure was used in this work except that it was found unnecessary to isolate and recrystallize the brucine formyl-L-methionine. Only the deviations from the methods of the above authors are described below.

The DL-methionine used for the resolution contained 9.42 per cent nitrogen; theoretical, 9.39 per cent. Formyl-DL-methionine, melting at 100–102° and containing the theoretical nitrogen content, was prepared by the method of Windus and Marvel.

30 gm. quantities of formyl-DL-methionine were used for each resolution. 50 gm. of recrystallized brucine were dissolved in the combined filtrates (6500 ml.) obtained from the original crystallization and the recrystallization of brucine formyl-D-methionine. The solution was allowed to stand at 0° for 5 days, during which time practically no crystallization occurred. The solution was then concentrated to about 3.5 liters and after cooling at 0° for 7 days 2.1 gm. of crystalline solid were removed by filtration and discarded. The filtrate was then concentrated and finally dried to a powder in a vacuum over calcium chloride. This solid was stirred for 30 minutes with 300 ml. of water, and the suspension was filtered with suction and washed with water on the filter. The insoluble brucine was dis-

carded. The solution, combined with the washings, was treated for the removal of brucine from the brucine formyl-L-methionine.

Methionine was obtained from the formyl isomers by hydrolysis with 10 per cent hydrochloric acid as described by Windus and Marvel (1). Analytically pure methionine was obtained by recrystallization of the hydrolysis product from 67 per cent ethanol.

Hydrochloric acid solutions used as solvent for the determinations of optical rotation were made from twice distilled, constant boiling hydrochloric acid which was standardized with pure sodium carbonate. The solutions of D- and L-methionine were filtered through a Pyrex bacterial filter to remove a trace of turbidity before determination of the rotation. Experiment showed that filtration did not lower the angular readings, but better definition was obtained with the filtered solutions. Rotations were carried out with a 2 dm. semimicro tube of 3 to 4 ml. capacity. Melting points were determined by the use of National Bureau of Standards calibrated Anschütz thermometers.

RESULTS AND DISCUSSION

The yields of the formyl isomers obtained by resolution of two 30 gm. quantities of formyl-DL-methionine and their melting points after each recrystallization are given in Table I. Purity rather than high yield was stressed. Windus and Marvel (1) and Jackson and Block (2) reported the melting points of formyl-DL-methionine and the D and L isomers of formyl-methionine as 98–100°. The dimorphous forms obtained in the first resolution (Run I) melted at 86–87.5° and 86–87° for the D and L isomers, respectively, when recrystallized to constant melting point. On repetition of the resolution (Run II) the formyl-D-methionine melted at the same temperature, but the L isomer melted at 100–101.5°. The low melting isomers are an unstable form. After they had stood at room temperature for 2 to 3 months, the formyl-D-methionine samples from Runs I and II had melting points of 100–101°, and the formyl-L-methionine from Run I had a melting point of 99–101°. There was, however, marked softening at 86–87°, after which there was no further change until the higher melting point was reached. The high melting form of formyl-L-methionine (Run II) did not soften at 86–87°, which indicated that it contained only the high melting form and that the transition from the low melting to the high melting form was not complete in 2 to 3 months.

Interconversion of the dimorphous forms of formyl-L-methionine by appropriate seeding of supersaturated solutions was attempted, but, after the high melting formyl-L-methionine was obtained, it was no longer possible to obtain the low melting form. This was due apparently to the presence in the laboratory of nuclei of the more stable form. On seeding a supersaturated solution of high melting formyl-L-methionine with crys-

tals of the low melting form, only the high melting form was obtained. The seed crystals used, however, were 6 weeks old and some of them prob-

TABLE I

Yields and Melting Points of Formylmethionine Isomers Obtained by Resolution of Two 30 Gm. Quantities of Formyl-DL-methionine

Recrystallization No.*	Formyl-D-methionine				Formyl-L-methionine			
	Run I		Run II		Run I		Run II	
	Yield	M.p.	Yield	M.p.	Yield	M.p.	Yield	M.p.
	gm.	°C.	gm.	°C.	gm.	°C.	gm.	°C.
0	8.2	86-88	8.0	84-86	5.8	86	8.4	100-101.5
1	6.8	85-86	5.6	86-86.5	4.1	86-87	6.9	100-101.5
2	5.6	86-87.5	3.8	86-87	3.1	86-87		
3	4.4	86-87.5	3.0	86-87.5				
4				86-87.5				

*Recrystallizations were from approximately 20 per cent solutions in ethyl acetate cooled to 5° or -5°.

TABLE II

*Optical Rotation and Nitrogen Content of Formyl-D- and L-Methionine and Respective D- and L-Methionine Preparations**

Isomer	Run I		Run II	
	Nitrogen†	$[\alpha]_D^{19-20\dagger}$	Nitrogen†	$[\alpha]_D^{19-20\dagger}$
	per cent	degrees	per cent	degrees
Formyl-D-methionine§	8.12	+11.4 ± 0.3	7.93	+11.4 ± 0.3
Formyl-L-methionine§	7.93	-11.1 ± 0.3	7.84	-11.8 ± 0.3
D-Methionine	9.39	+8.0 ± 0.3	9.46	+8.0 ± 0.3
L-Methionine	9.39	-7.8 ± 0.3	9.45	-8.0 ± 0.3

* The most highly purified samples described in Table I were used.

† The author is indebted to Dorris C. Chambers for the nitrogen determinations by the Kjeldahl micromethod.

‡ Water solutions containing 20 mg. per ml. were used.

§ Theoretical nitrogen, 7.91 per cent. Windus and Marvel reported $[\alpha]_D^{25} = +10.6^\circ$ and -10.0° , and Jackson and Block reported $[\alpha]_D^{25} = +12.2^\circ$ and -11.0° for the rotation of the D and L isomers, respectively.

|| Theoretical nitrogen, 9.39 per cent. Mueller reported $[\alpha]_D^{20} = -7.2^\circ$ for L-methionine isolated from casein. Windus and Marvel reported $[\alpha]_D^{25} = +8.12^\circ$ and -7.5° and Jackson and Block $[\alpha]_D^{25} = +7.0^\circ$ and $+7.2^\circ$ and -7.4° and -7.1° , for the rotations of the D and L isomers, respectively.

ably had changed to the high melting form; so this experiment was not conclusive.

When equal quantities of the enantiomorphic low melting isomers of

formyl-methionine were crystallized together, the resulting formyl-DL-methionine melted at 98–100.5°. In physical appearance, the low melting formyl-L-methionine was usually light and fluffy, while that of the high melting form was dense and granular.

The nitrogen contents and specific rotations of the pure formyl derivatives, together with the nitrogen contents and rotations of the methionine obtained from respective samples, are shown in Table II. The rotations

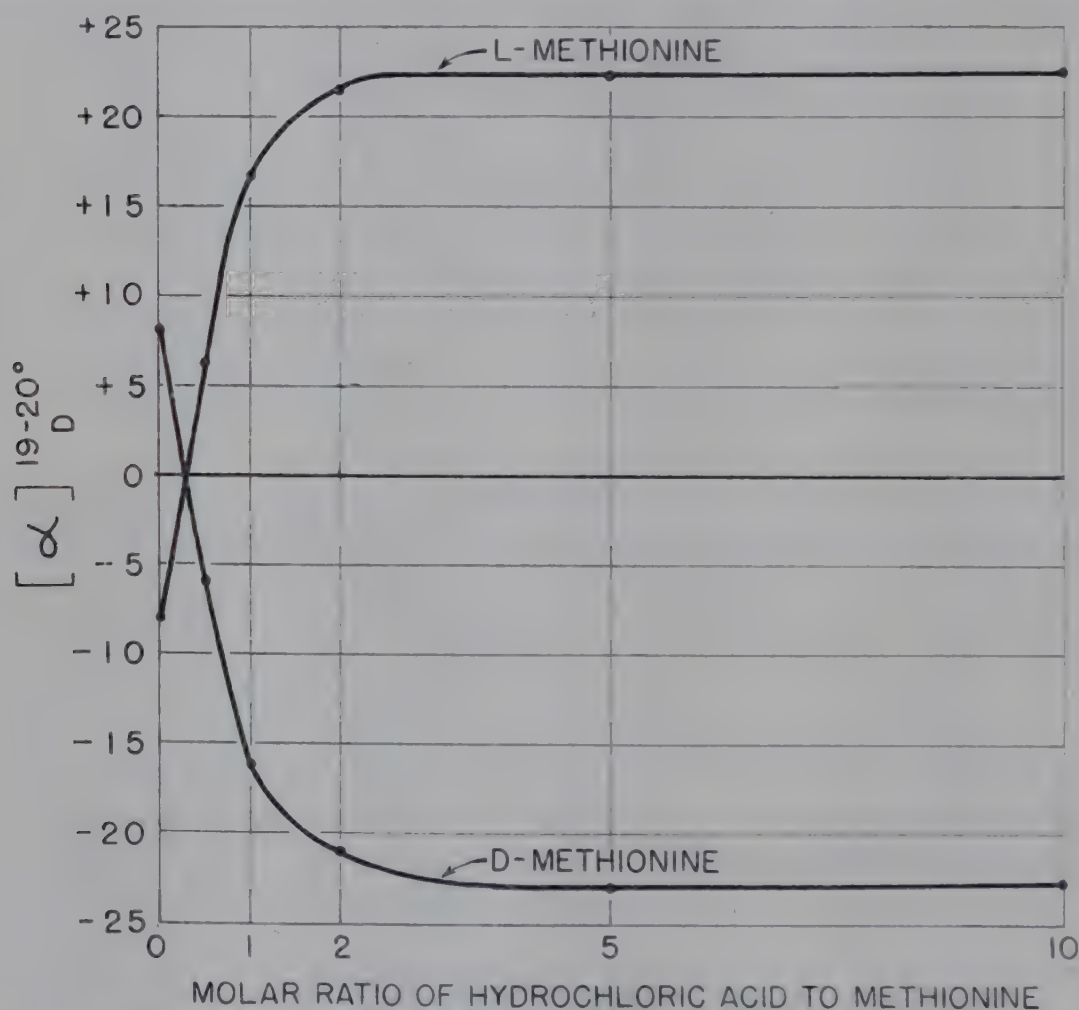


FIG. 1. Effect of hydrochloric acid on the optical rotation of D- and L-methionine. Solutions containing 100 mg. of methionine in 5 ml. of 0.067, 0.134, 0.268, 0.670, and 1.34 N hydrochloric acid were used to give molar ratios of 0.5, 1, 2, 5, and 10, respectively. The precision of the measurements was $\pm 0.3^\circ$.

of the enantiomorphous forms were equal but of opposite sign, and the values agreed well with those previously reported in the literature (1, 2). The rotation of the 86–87.5° formyl-L-methionine agreed well with that of the 100–101.5° form, and the rotations of the methionine obtained from each form also agreed well with each other and with values given in the literature (1–4).

The optical rotations of amino acids in hydrochloric acid have long been used as a test of their purity, and Lutz and Jirgensons (5, 6) showed that

the concentration of the amino acid as well as the molar ratio of amino acid to hydrochloric acid affects the values obtained. Windus and Marvel (1) and du Vigneaud and Patterson (4), respectively, reported $[\alpha]_D^{25} = -21.18^\circ$ and -22° for the rotation of D-methionine in 0.2 N hydrochloric acid, and Duschinsky and Jeannerat (7) reported $[\alpha]_D^{20} = +23.4^\circ$ for the rotation of 5 per cent L-methionine in the presence of 10 moles of hydrochloric acid. The effect of varying the concentration of hydrochloric acid on the optical rotation of the isomers of methionine has not been reported previously.

The effect of hydrochloric acid on the specific rotations of D- and L-methionine, obtained from Runs I and II, respectively, is shown in Fig. 1. The samples of methionine used were prepared by hydrolysis of formyl-D- and L-methionine which melted at 86–87.5° and 100–102°, respectively, as shown in Table II. The concentration of methionine was uniformly 20 mg. per ml., and the molar ratios of hydrochloric acid to methionine were 0.5, 1, 2, 5, and 10. The symmetry of the two curves, Fig. 1, shows that an excellent separation of the isomers of methionine was obtained. This was also shown by the coincidence of the values of the ratios of hydrochloric acid to methionine at 0.3 (0.04 N acid) at which the rotation of both forms was zero and also by the close agreement of the maximum rotation values ($+22.3^\circ$ and $+22.5^\circ$ and -23.0° and -22.8° for the L and D isomers, respectively) at 5 and 10 M ratios. The maximum values for the rotation of D- and L-methionine in hydrochloric acid agree well with those given in the literature (1, 7).

SUMMARY

A case of dimorphism of formyl-D- and L-methionine is described. The effect of hydrochloric acid on the optical rotation of D- and L-methionine has been determined.

BIBLIOGRAPHY

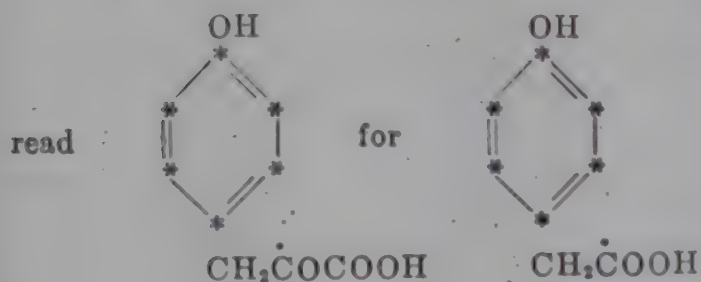
1. Windus, W., and Marvel, C. S., *J. Am. Chem. Soc.*, **53**, 3490 (1931).
2. Jackson, R. W., and Block, R. J., *J. Biol. Chem.*, **122**, 425 (1938).
3. Mueller, J. H., *J. Biol. Chem.*, **56**, 157 (1923).
4. du Vigneaud, V., and Patterson, W. I., *J. Biol. Chem.*, **109**, 97 (1935).
5. Lutz, O., and Jirgensons, B., *Ber. chem. Ges.*, **63**, 448 (1930).
6. Lutz, O., and Jirgensons, B., *Ber. chem. Ges.*, **64**, 1221 (1931).
7. Duschinsky, R., and Jeannerat, J., *Compt. rend. Acad.*, **208**, 1359 (1939).

CORRECTIONS

In the articles beginning on pages 1, 23, 33, and 43, Vol. 168, No. 1, April, 1947, and in the article beginning on page 11, Vol. 179, No. 1, May, 1949, read *Lactobacillus brevis* (8287) for *Lactobacillus brevis* (8257).

In Vol. 180, No. 3, October, 1949, on page 1154, legend to Fig. 6, read *Sample V* for *Sample VI*; on page 1155, legend to Fig. 7, read *Sample VI* for *Sample VII*; on page 1156, second paragraph, read *fasting 18 hours and 4 hours* for *fasting 4 hours and 18 hours*.

In Vol. 181, No. 1, November, 1949, on page 2S2, third structure,



SPONTANEOUS OCCURRENCE OF AN UNIDENTIFIED PIGMENT IN ERYTHROCYTES OF RABBITS FED A PURIFIED DIET

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In the course of studies on metabolism of erythrocytes of rabbits maintained on a purified diet, it was observed that the washed red cells of an occasional animal had a darker appearance than usual. This apparent aberration was investigated by comparing the absorption spectra of hemolysates from the rabbits on a purified diet and controls on a laboratory stock diet. The results provide evidence for the occurrence of an abnormal pigment that is retained in the washed erythrocytes from rabbits fed a purified diet.

Procedure

An inbred strain of white New Zealand rabbits was used in these experiments. Weanling male litter mates weighing about 1700 gm. were fed a purified ration designated as Diet 672 as previously described (1). The controls received the ration fed the breeding colony.¹ Diet 672, although able to maintain many of the rabbits for a period of years, is not adequate for maximal growth, reproduction, and longevity. The animals were about 1 year of age at the time they were bled for this study. The erythrocytes were washed four times in 0.85 per cent NaCl and were then packed by a standard centrifugation. They were hemolyzed by diluting 1:20 in 0.02 M buffer of appropriate pH containing 0.3 per cent freshly dissolved saponin for analysis in the red and near infra-red region of the spectrum. 1 ml. of this solution was diluted to 25 ml. with 0.02 M buffer for readings in the visible region. The hemolysate was cleared by centrifugation and equilibrated with an O₂ or CO atmosphere for 20 minutes, or the pigment was converted to methemoglobin (MHB) with a small crystal of K₃Fe(CN)₆ and subsequently to cyanmethemoglobin (MHB-CN) by addition of a crystal of KCN. The absorption spectra measured on the Beckman spectrophotometer were plotted as millimolecular extinction

¹ The stock ration contained alfalfa leaf meal 40 per cent, pulverized No. 1 whole wheat 30.5 per cent, oat groats 15 per cent, soy bean meal 13 per cent, and supplements of calcium carbonate, iodized sodium chloride, irradiated yeast, and wheat germ oil, all of which was pressed into BB size pellets.

coefficients ($\epsilon \times 10^{-3}$). The latter term indicates the light absorption from a solution containing 1 mM of hemoglobin Fe per liter. In order to compute the coefficients the hemoglobin concentration was determined by diluting 1.0 ml. of the 1:20 hemolysate dilution with 24 volumes of 0.02 M buffer and reading at 540 m μ after conversion to MHbCN. The extinction coefficients in the red region were then calculated by employing Drabkin's constant (2) and allowing for dilution according to the relationship

$$\epsilon = \frac{d_1 \times 11.5 \times 10^{-3}}{d_2 \times 25}$$

where d_1 is the density ($\log_{10} I_0/I$) of the 1:20 dilution at a given spectral interval and d_2 is the density at 540 m μ of the 1:500 dilution after conversion to MHbCN.

Results

While studying methemoglobin disappearance after the addition of nitrite to red blood cells of rabbits, it was noted that a dark color persisted in the hemolysates of certain animals after the MHb had largely disappeared. This was encountered in erythrocytes from two litter mates and later in two additional animals maintained on Diet 672. Examination of hemolysates from these rabbits in the spectrophotometer showed increased optical density in the red, maximal at 615 to 625 m μ . This increased light absorption was observed despite the presence of cyanide. When examined in the visible region, these hemolysates showed spectra with slightly greater absorption than the controls at both extremes and somewhat decreased absorption at the maxima.

Subsequently a group of rabbits available on the experimental diet was surveyed for the presence of the spectral deviation. Hemolysates from fifteen animals fed the purified ration and seventeen animals fed the laboratory stock diet were examined spectroscopically after centrifugation and equilibration with O₂. Fig. 1 shows the range of individual variation obtained in the hemolysate spectra of the seventeen controls between 600 and 650 m μ and the curves of seven of the fifteen experimental animals which fall outside the normal range. The different contour of the curve when low levels of MHb are present in similarly treated hemolysate pooled from three control animals is also shown in Fig. 1. The increased absorption encountered a week later in the hemolysate of Rabbit E-2 equilibrated against an atmosphere of CO is evident in Fig. 2. It is to be noted that hydrosulfite failed to alter the CO curves as it would have had the solutions contained MHb. Fig. 2 also shows the more uniformly increased absorption in the red region in the hemolysate from the experimental

animal when the pigment is converted to alkaline MHB or MHB_{CN}. A similarly increased optical density was noted in Rabbit E-2 hemolysate at 500 to 520 $m\mu$ on examination of these solutions in the visible region.

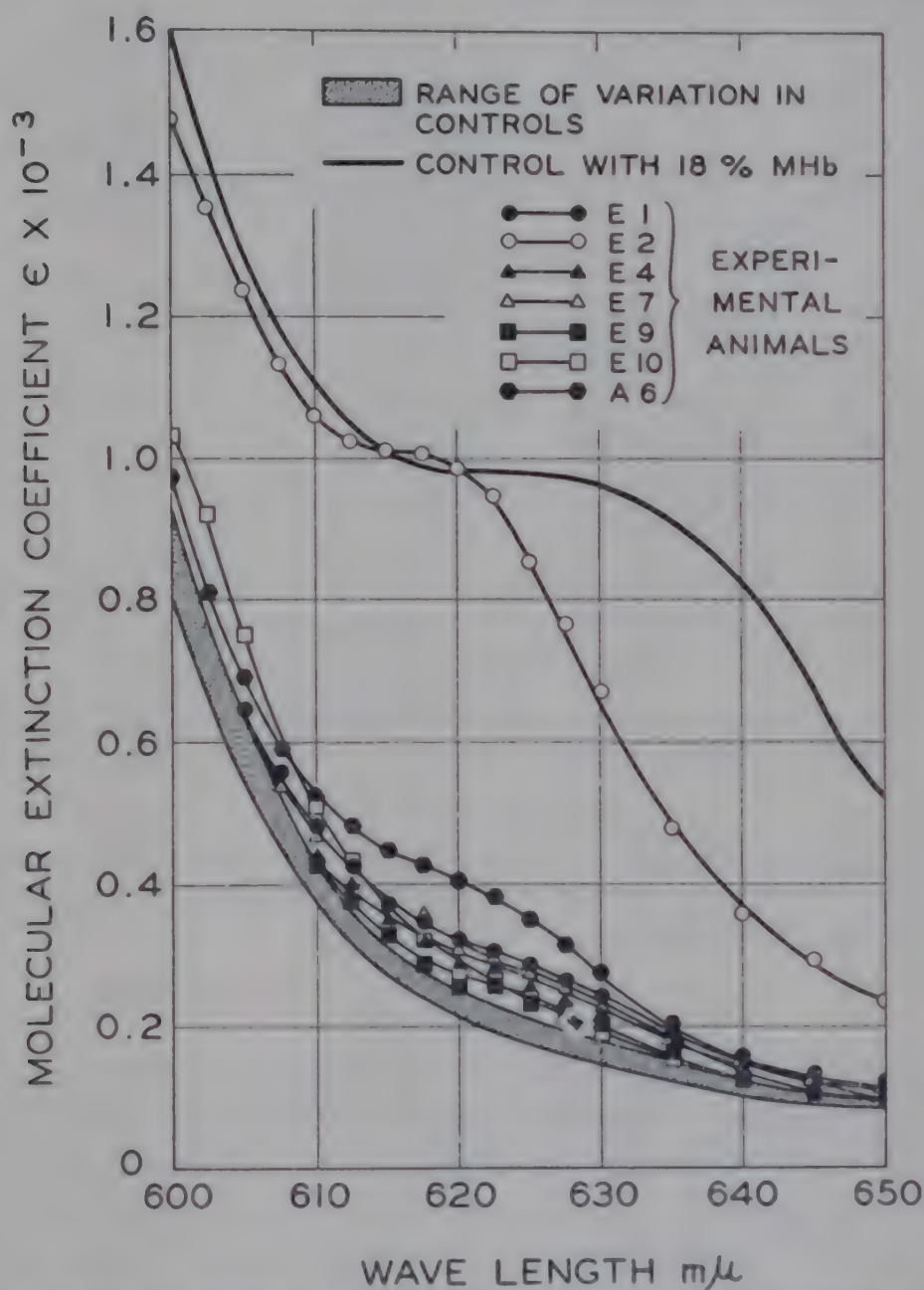


FIG. 1. Absorption spectra of oxygenated hemolysates of erythrocytes in the interval 600 to 650 $m\mu$.

After equilibration with an atmosphere of N_2 the hemolysate of Rabbit E-2, at a time when the deviation of the oxy form at 620 $m\mu$ had decreased to one-half its previous intensity, showed no convexity in this area, but had a uniformly greater absorption than the control, resembling the relationship of the MHB derivatives in Fig. 2.

By subtracting the mean extinction coefficients of the oxygenated hemolysates of the control rabbits from the coefficients of the oxygenated

hemolysate of either Rabbit E-2 or A-6, a curve was obtained showing a maximum at 620 $m\mu$. A curve for the CO derivative having a maximum at 615 $m\mu$ was obtained by subtracting the values for ϵ of a control animal from those of Rabbit E-2.

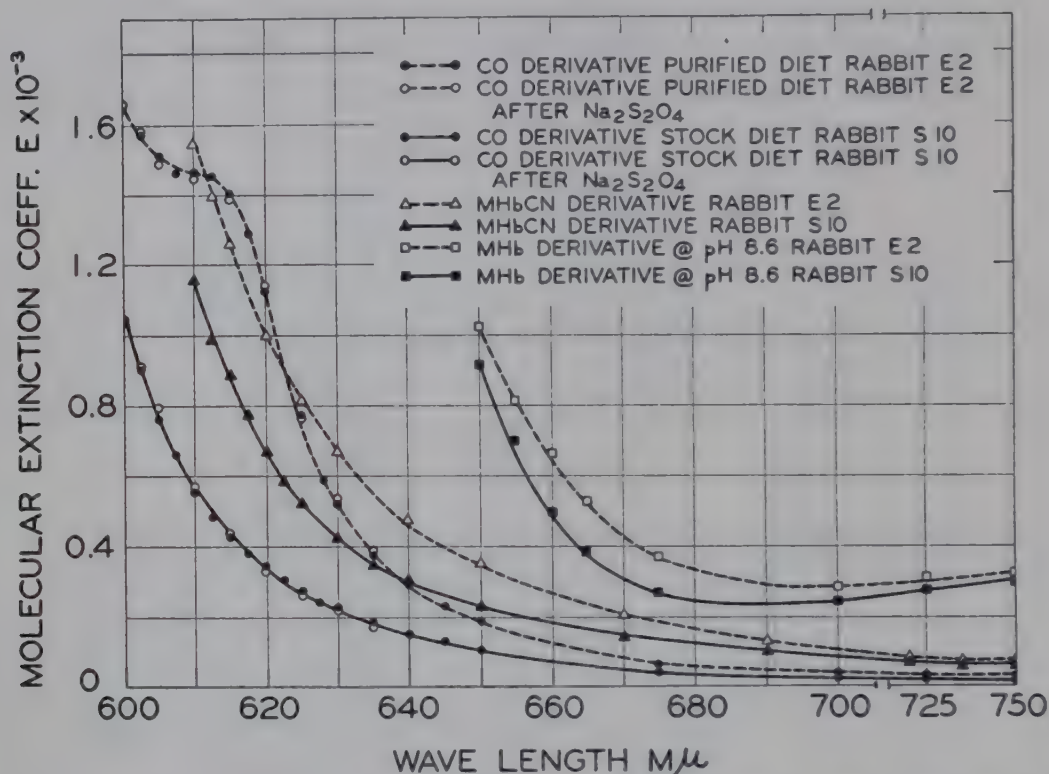


FIG. 2. Absorption spectra in the near infra-red region of the alkaline MHb, MHbCN, and CO derivatives of hemolysates of erythrocytes from rabbits on a purified diet and a control ration.

DISCUSSION

The pigment causing the dark color of the hemolysates of rabbits fed a synthetic diet differs in several ways from MHb (3). Its presence in washed cells would serve to differentiate it from dialyzable substances such as heme and porphyrin. The positions of its maxima are distinct from those of choleglobin (verdoglobin A), the oxidation product of hemoglobin which was observed *in vitro* by Lemberg (4). However, in respect to the position of the absorption bands of the oxy and CO derivatives and the resistance to reduction by $Na_2S_2O_4$ the pigment in question is not dissimilar to sulfhemoglobin (verdoglobin S) (4-7). On the other hand, it differs from the sulfhemoglobin (SHb) observed by Drabkin in a case of acetanilide poisoning (8) in that the reduced and cyanmet forms are obtainable. Again the pigment concerned here is unlike the SHb induced in rabbits by adding phenacetin and sulfur to the diet, since the latter substance after oxidation at an alkaline pH showed a maximum at 620 $m\mu$ (7).

SUMMARY

A grossly evident dark color has been encountered in washed erythrocytes of rabbits fed a purified diet for a period of from 1 to 2 years. Spectroscopic examination of hemolysates of these cells revealed the presence of an abnormal pigment, the characteristics of which distinguish it from the known derivatives of hemoglobin. The red blood cells of slightly less than half the animals on the experimental diet but of none of those on the stock diet gave spectroscopic evidence of the pigment.

BIBLIOGRAPHY

1. Wooley, J. G., and Sebrell, W. H., *J. Nutr.*, **19**, 191 (1945).
2. Drabkin, D. L., *Am. J. Med. Sc.*, **209**, 268 (1945).
3. Horecker, B. L., *J. Biol. Chem.*, **148**, 173 (1943).
4. Lemberg, R., Legge, J. W., and Lockwood, W. H., *Biochem. J.*, **33**, 754 (1939).
5. Hoppe-Seyler, F. C., *Centr. med. Wissensch.*, **1**, 433 (1863).
6. Nijveld, H. A. W., *Rec. trav. chim. Pays-Bas*, **62**, 293 (1943).
7. Kiese, M., and Kaeske, H., *Biochem. Z.*, **312**, 121 (1942).
8. Drabkin, D. L., and Austin, J. H., *J. Biol. Chem.*, **112**, 51 (1935-36).

ENZYMATIC RESOLUTION OF RACEMIC LYSINE, NORLEUCINE, NORVALINE, AND α -AMINOBUTYRIC ACID

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The asymmetric action of hog kidney and beef pancreas preparations on racemic *N*-acylated amino acids has led to the development of a rapid enzymatic method for the resolution of several amino acids in good yield and with a high degree of optical purity (1-3). The optical isomers of the following amino acids have been prepared: alanine, valine, leucine, isoleucine, methionine, serine, threonine, phenylalanine, tyrosine, tryptophan, and aspartic and glutamic acids. The present communication describes the extension of this method to the resolution of racemic lysine, norleucine, norvaline, and α -aminobutyric acid.

The only reported resolution of lysine is that of Berg (4), who employed a fractional crystallization of the isomeric lysine camphorates in 50 per cent methanol. Norleucine (5) and α -aminobutyric acid (6) were resolved by the Fischer school through the cinchonine and morphine salts, respectively, of the corresponding *N*-benzoyl derivatives. To our knowledge, norvaline has not yet been resolved.

EXPERIMENTAL

The starting materials were the *N*-chloroacetylated derivatives of the respective amino acids, prepared in the usual manner in sodium hydroxide solution with chloroacetyl chloride.¹ Acidification of the reaction mix-

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¹ Other derivatives of racemic lysine prepared for this study included ϵ -carbobenz-oxylysine (m.p. about 245°; N calculated 10.0, found 9.9), which was synthesized in the same manner as the optically active compound (7) and which was chloroacetylated to give in 50 per cent yield ϵ -carbobenzoxy- α -chloroacetyl-DL-lysine. After crystallization as needles from acetone, the melting point was 114°; N calculated 7.8, N found 7.6. On catalytic hydrogenation with palladium black, α -chloroacetyl-DL-lysine was obtained, which, after crystallization as needles from 50 per cent alcohol, possessed a melting point of 226°, and N calculated 12.6, N found 12.4. A sample of ϵ -benzoyl- α -chloroacetyl-DL-lysine was kindly donated by Dr. J. White. When tested for susceptibility to the action of crude hog kidney extract, the rate of hydrolysis of all of the α -chloroacetylated lysine derivatives was about 6 to 10 μ M

ture led to the precipitation of the norleucine and norvaline derivatives, which, after drying over phosphorus pentoxide, were recrystallized as needles from acetone-ether. The corresponding derivatives of α -aminobutyric acid and of lysine did not precipitate from the reaction mixture on acidification, and were therefore extracted several times with ethyl acetate; the extracts were combined, dried, and evaporated *in vacuo* to dryness. Chloroacetyl-DL- α -aminobutyric acid crystallized quickly and was recrystallized from acetone-ether. α,ϵ -Dichloroacetyl-DL-lysine, however, crystallized only after standing 3 to 4 months at 5°; subsequent preparations were obtained in crystalline form in a few hours by the use of seed crystals. This compound was also recrystallized from acetone-ether mixtures from which it separates as spongy masses of needles. Analytical data on these compounds are as follows: chloroacetyl-DL-norleucine (m.p. 116°; N calculated 6.8, found 6.8), chloroacetyl-DL-norvaline (m.p. 101°; N calculated 7.3, found 7.2), chloroacetyl-DL- α -aminobutyric acid (m.p. 128°; N calculated 7.8, found 7.7), and α,ϵ -dichloroacetyl-DL-lysine (m.p. 103°; N calculated 9.3, found 9.3). For purposes of comparison ϵ -chloroacetyl-amino-*n*-caproic acid (m.p. 84°, N calculated 6.8, found 6.8) was prepared from ϵ -amino-*n*-caproic acid and chloroacetyl chloride in alkaline solution. In similar fashion, chloroacetyl- β -alanine (m.p. 98°, N calculated 8.5, found 8.5) was prepared.

The rates of hydrolysis in terms of micromoles of L form cleaved per hour per mg. of N at pH 7.0 and 38° by crude hog kidney aqueous extracts were, for the chloroacetylated derivatives of racemic norleucine 1070, norvaline 1600, α -aminobutyric acid 1111, and lysine 10.² ϵ -Chloroacetyl-amino-*n*-caproic acid and chloroacetyl- β -alanine were completely resistant.

The need for a concentrated preparation of the hog kidney enzyme was clearly indicated for the resolution of the relatively resistant lysine derivative. A description of this preparation has been given (1, 2). That

per hour per mg. of N at pH 7.0 and 38°. There was no advantage in the use of these compounds over that of the dichloroacetyl derivative for the resolution procedure, and, indeed, because of the low solubility of the ϵ -carbobenzoxy or benzoyl derivatives, the task of separating them from the large amount of protein needed in the enzymatic digests would be likely to be onerous.

² The L form of such amino acids as norvaline and α -aminobutyric acid, for which the natural isomer is not known or available, is assumed to be that form whose N-acyl derivative is hydrolyzed by the kidney enzyme. The rate values are taken from the initial linear portion of the hydrolysis time curves up to 30 per cent hydrolysis of the susceptible form of the substrate (1-3), with the ninhydrin-CO₂ manometric method for following the hydrolysis. Digests consisted of 1 cc. of extract, 1 cc. of phosphate buffer at pH 7.2, and 1 cc. of either water or 0.05 M neutralized substrate.

employed for the present studies represented a 6-fold increase in activity over the initial crude extract, as measured by the rate of hydrolysis of chloroacetyl-DL-alanine (*cf.* (2)).

The resolution of norleucine, norvaline, and α -aminobutyric acid by means of the hog kidney preparation followed exactly the procedure described earlier for leucine, valine, etc. (2). About 15 cc. of the enzyme mixture were used for the resolution of 100 gm. each of the chloroacetylated derivatives. The reaction was completed in 2 to 4 hours at 38°,

TABLE I
*Specific Optical Rotations at 23° of Amino Acids**

Amino acid	Present data					Data in literature†	
	L Form		D Form		N calcu- lated	L Form	D Form
	Rotation	N found	Rotation	N found			
	<i>degrees</i>		<i>degrees</i>			<i>degrees</i>	<i>degrees</i>
α -Aminobutyric acid.....	+20.6‡	13.6	-20.7‡	13.6	13.6	+19.5 (6)§	-19.5 (6)§
Norvaline.....	+24.8	12.0	-24.8	12.0	12.0		
Norleucine.....	+23.3	10.7	-22.8	10.7	10.7	+21.3 (5)	-22.4 (5)
Lysine.....	+23.0¶	12.6	-23.1¶	12.6	12.8	+23.6 (4)**	-23.6 (4)**

* As before (1-3), all the measurements were made in a 2 dm. tube.

† The figures in parentheses are bibliographic references.

‡ 4.00 per cent solutions in 6 N HCl.

§ Figures taken from the table by D. M. Greenberg (8), recalculated on the basis of the free amino acid from data on the hydrochloride salts.

|| 4.15 per cent solutions in 6 N HCl.

¶ 4.00 per cent solutions of the respective dihydrochlorides in 6 N HCl; the measured specific rotations were +15.2° and -15.3°, respectively. The data in Table I were calculated from these figures on the basis of the free amino acid.

** The data given by Berg (4) are for L-lysine (+15.63°) and D-lysine (-15.65°) dihydrochlorides at 3.00 per cent solutions in water. The figures given in Table I have been calculated approximately for the free amino acid.

but was allowed to proceed for 16 hours. The yields of the individual isomers were of the same order as for the other amino acids; *e.g.*, about 70 per cent of the L form and about 60 per cent of the D form.³ The analytical and optical data are given in Table I. The resolution of lysine necessitated a few slight variations, since an intermediate in the preparation of the L isomer was isolated, and is therefore described in more detail.

L-Lysine Dihydrochloride—70 gm. of recrystallized α,ϵ -dichloroacetyl-

³ Based upon the amount of chloroacetylated racemic amino acid taken initially.

DL-lysine were suspended in 80 cc. of chilled, distilled water, and dissolved by the dropwise addition of 6 N lithium hydroxide, until the pH was 7.6. 200 cc. of the enzyme preparation were added, the pH again adjusted to 7.6, and the mixture allowed to digest at 38°. As usual (1-3), aliquots were removed from time to time to follow the course of the digestion, and were analyzed by the ninhydrin-CO₂ manometric procedure. After about 32 hours, the reaction appeared to have halted with the hydrolysis of one bond of one of the isomeric forms.⁴ No hydrolysis of the ϵ -chloroacetyl-amino group was to have been expected, in view of the lack of susceptibility of ϵ -chloroacetyl-amino-*n*-caproic acid (see above), and presumably the bond hydrolyzed was that uniting the α -chloroacetyl residue to the L isomer. In order to be certain of the completion of the hydrolysis, the pH of the mixture was again adjusted to 7.6, 50 cc. more of the enzyme preparation were added, and the digestion allowed to proceed for another 16 hours (1-3). The mixture was then acidified to pH 5.0 with glacial acetic acid, shaken with norit for 1 hour, and filtered by suction through a thin layer of norit. The filtrate was evaporated *in vacuo* to a small volume and treated with an excess of hot absolute ethyl alcohol. The compound which quickly crystallized was ϵ -chloroacetyl-L-lysine. It was filtered and washed several times with hot alcohol. The mother liquor and washings were set aside for the preparation of the D isomer of lysine. The ϵ -chloroacetyl-L-lysine was admixed with a small amount of protein, and was therefore recrystallized twice from hot water-alcohol mixtures with the aid of norit. The final product appeared as rosettes of fine needles and was protein-free. The yield of crude material was 22 gm., or 78 per cent of the theory based upon the dichloroacetyl compound. The dried, pure compound yielded the following analysis: (C₈H₁₅O₃N₂Cl), N calculated 12.6, found 12.4. $[\alpha]$ at 23° for a 4.00 per cent solution in 6 N HCl = +23.1° (2 dm. tube).

11 gm. of this compound were refluxed with 220 cc. of 2 N HCl for 2

⁴The amount of enzyme preparation used should have been sufficient to complete the hydrolysis in about 10 hours if the initial reaction rate of 10 μ M per hour per mg. of N, multiplied by 6 for the concentration of activity, had held throughout the reaction. The rate, however, as is usual in enzymatic reactions, falls off with time and approaches complete hydrolysis asymptotically. The value for the initial reaction rate serves only as a rough guide to the decision as to how much of the enzyme preparation to use. The nearly equal absolute values for the rotation of the two isomers of each of the amino acids so far studied (1-3) (Table I) shows that the enzymatic hydrolysis of the acylated-L form reached completion, while the acylated-D form was completely resistant. Manometric ninhydrin analyses of the enzyme preparations show almost negligible α -amino acid blanks. The maximum possible impurity from this source may vary from 0.2 per cent for lysine, whose resolution requires much enzyme, to less than 0.02 per cent for alanine or serine, whose resolution requires little enzyme.

hours, treated with norit, evaporated to dryness *in vacuo*, and rubbed with cold absolute alcohol to crystallization. After standing for several hours at 5° with an excess of acetone, the crystals were filtered, washed with acetone and ether, and dried. The dried product was pulverized, rubbed with cold absolute alcohol, filtered, washed with ether, and dried. This second alcohol wash is necessary in order to remove the last trace of faint yellow color from the compound. The yield of pure L-lysine dihydrochloride so obtained was about 80 per cent of the theory, based upon the ϵ -chloroacetyl compound. The analytical and optical data are given in Table I.

D-Lysine Dihydrochloride—The mother liquor and washings from the preparation of the ϵ -chloroacetyl-L-lysine were combined and evaporated *in vacuo* nearly to dryness. The residue was taken up in about 30 cc. of water, acidified to pH 1.7 with concentrated hydrochloric acid, and extracted six times with ethyl acetate. The extracts were combined, dried over sodium sulfate, the filtrate evaporated *in vacuo* to dryness, and the residual syrup washed several times with petroleum ether. The washed residue was refluxed with 250 cc. of 2 N hydrochloric acid for 2 hours. D-Lysine dihydrochloride was isolated from the hydrolysate and worked up in the same fashion as described for the L form. The yield was 65 per cent of the theory, based upon the initial amount of racemic dichloro-acetyllysine used. The characteristics of the compound are given in Table I.

Attempts to Resolve Other Amino Acids—We have so far been able to resolve simply and conveniently most of the naturally occurring amino acids (1-3) (Table I). The chief remaining representatives of this class of substances are proline, hydroxyproline, cystine, arginine, and histidine. N-Acetylproline is completely resistant to the action of hog kidney (1), as are other compounds lacking a peptide hydrogen such as chloroacetyl-sarcosine and chloroacetyl-N-methylalanine (9), and it appears doubtful whether proline (or hydroxyproline) can be resolved by the present enzymatic method. Dichloroacetyl-L-cystine (m.p. 136°, N calculated 7.1, found 7.1) and N-acetyl-S-benzyl-DL-cysteine (m.p. 157°, N calculated 5.5, found 5.5) were prepared and subjected to the action of hog kidney and crystalline carboxypeptidase preparations. Surprisingly, these compounds were practically completely resistant to enzymatic attack from these sources, and it therefore does not seem likely that the present method is applicable to the resolution of cystine.

In order to resolve arginine and histidine by this method it is necessary that both basic groups on the respective amino acids be acylated, as was the case with lysine. Although dibenzoylarginine has been prepared (10), the N-benzoylamino group on the α -carbon is very weakly susceptible

to enzymatic attack, as compared with the *N*-chloroacetyl-amino or even the *N*-acetyl-amino group (2). Perbenzoylation of histidine results in an opening of the imidazole ring (11, 12). The masking of the basic groups of histidine and arginine by suitable substituents is at present under investigation.

The authors are indebted to Mr. Robert Koegel for the nitrogen analyses.

SUMMARY

1. The L and D isomers of norleucine ($+23.3^\circ$ and -22.8°), norvaline ($+24.8^\circ$ and -24.8°), and α -aminobutyric acid ($+20.6^\circ$ and -20.7°), measured in 6 N HCl, have been obtained by the asymmetric hydrolysis by hog kidney of the *N*-chloroacetylated derivatives of the respective racemic amino acids. The yields were about 70 per cent for the L, and about 60 per cent for the D form.

2. L- and D-lysine ($+23.0^\circ$ and -23.1°) were obtained in substantially the same fashion from α,ϵ -dichloroacetyl-DL-lysine. ϵ -Chloroacetyl-L-lysine was isolated in the course of the preparation of L-lysine. The yields of L- and D-lysine, obtained as the dihydrochlorides, were about 70 and 65 per cent, respectively.

3. ϵ -Chloroacetyl-amino-*n*-caproic acid, as well as dichloroacetyl-L-cystine and *N*-acetyl-S-benzyl-DL-cysteine, is not hydrolyzed by hog kidney preparations.

BIBLIOGRAPHY

1. Fodor, P. J., Price, V. E., and Greenstein, J. P., *J. Biol. Chem.*, **178**, 503 (1949).
2. Price, V. E., Gilbert, J. B., and Greenstein, J. P., *J. Biol. Chem.*, **179**, 1169 (1949).
3. Gilbert, J. B., Price, V. E., and Greenstein, J. P., *J. Biol. Chem.*, **180**, 473 (1949).
4. Berg, C. P., *J. Biol. Chem.*, **115**, 9 (1936).
5. Fischer, E., and Hagenbach, R., *Ber. chem. Ges.*, **34**, 3764 (1901).
6. Fischer, E., and Mouneyrat, A., *Ber. chem. Ges.*, **33**, 2383 (1900).
7. Bergmann, M., Zervas, L., and Ross, W. F., *J. Biol. Chem.*, **111**, 245 (1935).
8. Greenberg, D. M., in Schmidt, C. L. A., *Chemistry of amino acids and proteins*, 2nd edition, Springfield and Baltimore, 1177 (1944).
9. Fodor, P. J., Price, V. E., and Greenstein, J. P., *J. Biol. Chem.*, **180**, 193 (1949).
10. Felix, K., and Dirr, K., *Z. physiol. Chem.*, **176**, 29 (1928).
11. Kossel, A., and Edlbacher, S., *Z. physiol. Chem.*, **93**, 396 (1915).
12. Greenstein, J. P., *J. Biol. Chem.*, **109**, 529 (1935).

EFFECT OF THYMUS NUCLEATE ON THE THERMAL COAGULATION OF ALBUMIN SOLUTIONS

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Relatively small amounts of sodium thymus nucleate can apparently prevent the coagulation of egg albumin in salt-free solutions heated at 98° for several hours (1). As the original observations were based largely on visual estimations, we have recently applied more quantitative nephelometric determinations to the study of this protective phenomenon (2), and have extended such studies to horse and bovine serum albumin solutions. With all three proteins, the degree of heat stability conferred is proportional to the amount of nucleate added.

EXPERIMENTAL

Three preparations of chicken egg albumin, each crystallized several times, were donated for these studies by Dr. Gerson Kegeles. These preparations were designated Preparations I, II, and III, respectively, and had stood at 5° in the crystalline state over half saturated ammonium sulfate for several months. Horse serum albumin was prepared from fresh serum and crystallized three times. The crystalline bovine serum albumin was an Armour product.

The sodium thymus nucleate was prepared by the Hammarsten method (3) with the modifications described (4). Nucleate solutions were dialyzed for several days at 5° against distilled water. Unless both albumin and nucleate solutions are practically salt-free, the protective effect of the nucleate is not obtained. As shown earlier, addition of sodium chloride in relatively small amounts before the heating abolishes the otherwise protective effect of the nucleate against heat coagulation of the proteins (1).¹

Effect of Dialysis—Aqueous, dialyzed solutions of freshly crystallized chicken egg albumin or horse or bovine serum albumin, at the isoelectric point (pH 4.6 to 4.8), coagulate at 98° to a heavy mass of insoluble pro-

¹ The effect of the salt is demonstrated only when the mixture of protein, nucleate, and salt is heated together. Addition of sodium chloride to a heated and protected mixture of protein and nucleate produces no coagulation of the protein. Nucleate added alone to a coagulated albumin solution gives no clearing of turbidity.

tein with increase in pH to about 5.0. Addition of large amounts of thymus nucleate to the protein solutions before heating, up to 5 mg. of nucleate to 20 mg. of protein, did not prevent the subsequent heat coagulation of the protein. When the pH of the albumin solution was raised to 6.8 by addition of dilute sodium hydroxide, the protein did not coagulate on heating, and the solution remained clear. Subjection of such neutralized (unheated) solutions to prolonged dialysis against distilled water (pH 5 to 6) in the cold resulted in a lowering of the pH to the neighborhood of 5.2 to 5.4. These solutions then coagulated to a heavy turbidity on being heated at 98°, the pH rose in every case to 6.5 to 6.7, and, if sufficient thymus nucleate was added prior to the heating, complete protection against coagulation was achieved. The nucleate itself is not a buffer in this pH range, and its solutions, especially the very dilute ones used throughout these studies, mixed with those of the albumins without appreciable change in pH either before or after heating.

The reason for the decrease in pH of the neutralized albumin solutions during dialysis is obscure, particularly since simple heating of the dialyzed solutions restores nearly the initial pH. The pH drop is obviously not due to appreciable loss of alkali on dialysis, but may be due to combination with volatile acid such as carbonic acid. The dialyzed albumin thereby becomes heat-coagulable, like isoelectric albumin, but, unlike the latter, is protectable against such heat coagulation by added thymus nucleate. Whatever the significance of dialysis and the pH changes on the albumin coagulation phenomena may be, the nucleate effect is produced without significant pH alteration and therefore constitutes a separate problem.

The protein solutions employed were therefore prepared as follows. The crystalline albumins were suspended in cold distilled water, dilute NaOH was added until the pH of the resulting solutions was 6.8, and the solutions were dialyzed against frequent changes of cold distilled water until free from ammonia and sulfate ions. The clear solutions were then concentrated in cellophane sacs by a vigorous air current to the desired concentrations. Aliquots of such solutions (pH 5 to 6) at a concentration of 1 per cent, when plunged into boiling water, quickly yielded a heavy turbidity without separation of protein and lent themselves readily to nephelometry (2).

Effect of Varying Concentrations of Nucleate on Thermal Stability of Albumin Solutions—The horse and bovine serum albumin solutions were used at 2.3 per cent concentration, as were chicken egg albumin Preparations I and III. Egg albumin Preparation II was at 1.6 per cent concentration. The test mixtures were prepared by mixing 2 cc. of albumin solutions with 0.1 to 1 cc. amounts of nucleate (in 0.1 cc. quantities) and

enough water to give a total volume of 4 cc. in each case. The concentration of the original nucleate solution, 0.023 to 0.058 per cent, was so adjusted (with distilled water) that a continuous change in degree of turbidity would be produced by heating within the range of serial dilutions employed. This was generally approximated by noting the lowest concentration of nucleate which would afford practically complete protection of the albumin against heat coagulation, and beginning the serial dilutions from this concentration.

The control mixture consisted of 2 cc. of the albumin solutions plus 2 cc. of distilled water. Both control and test mixtures were simultaneously plunged into a boiling water bath, held there for 10 minutes, cooled to 25°, and the degree of turbidity in the test mixtures estimated nephelometrically against the control, which was taken as 100. Both test and control possessed practically the same pH (6.5 to 6.7) after heating.

The horse serum albumin solution was divided into three parts, and each was studied against different initial concentrations of nucleate; namely, 0.046, 0.031, and 0.023 per cent respectively. The bovine serum albumin was studied against a single nucleate concentration; namely, 0.046 per cent. The data are given in Fig. 1.

Preparation I of the chicken egg albumin solution was also divided into three parts, and studied against 0.092, 0.058, and 0.046 per cent nucleate respectively. Preparations II and III were studied against 0.04 and 0.058 per cent nucleate respectively. The data are given in Fig. 2.

The following observations may be noted: (a) The decrease in turbidity of the heated albumin solutions is approximately linear with increasing nucleate to about 10 to 25 per cent turbidity when it slopes off asymptotically toward the stage of nearly complete protection against coagulation; (b) with very small amounts of nucleate, the turbidity of the test mixtures is apparently higher than that of the controls, and (c) nearly complete protection of the albumin solutions against heat coagulation is afforded by ratios of approximately 100 to 200 mg. of protein to 1 mg. of nucleate. Actually, it is difficult to determine the last point mentioned because of the asymptotic character of the curves (Figs. 1 and 2), but the order of magnitude given presents some idea of the remarkable effect produced by the nucleate. The curves for the three aliquots of the horse serum albumin preparation (Fig. 1) and for the chicken egg albumin Preparation I (Fig. 2) illustrate the reproducibility of the data. It would have been desirable, in comparing the effect of the nucleate on the three albumins from different species, to have taken the 50 per cent turbidity values as the point of comparison. However, the fact that the curves at low nucleate concentrations do not extrapolate to the 100 per cent turbidity value of the control renders such a comparison dubious.

Effect of Desoxyribonuclease- Mg^{++} on Protected Mixtures—It was noted earlier (1) that, when a crude rat liver aqueous extract was protected against heat coagulation by addition of thymus nucleate, subsequent addition of a desoxyribonuclease preparation plus magnesium ions pro-

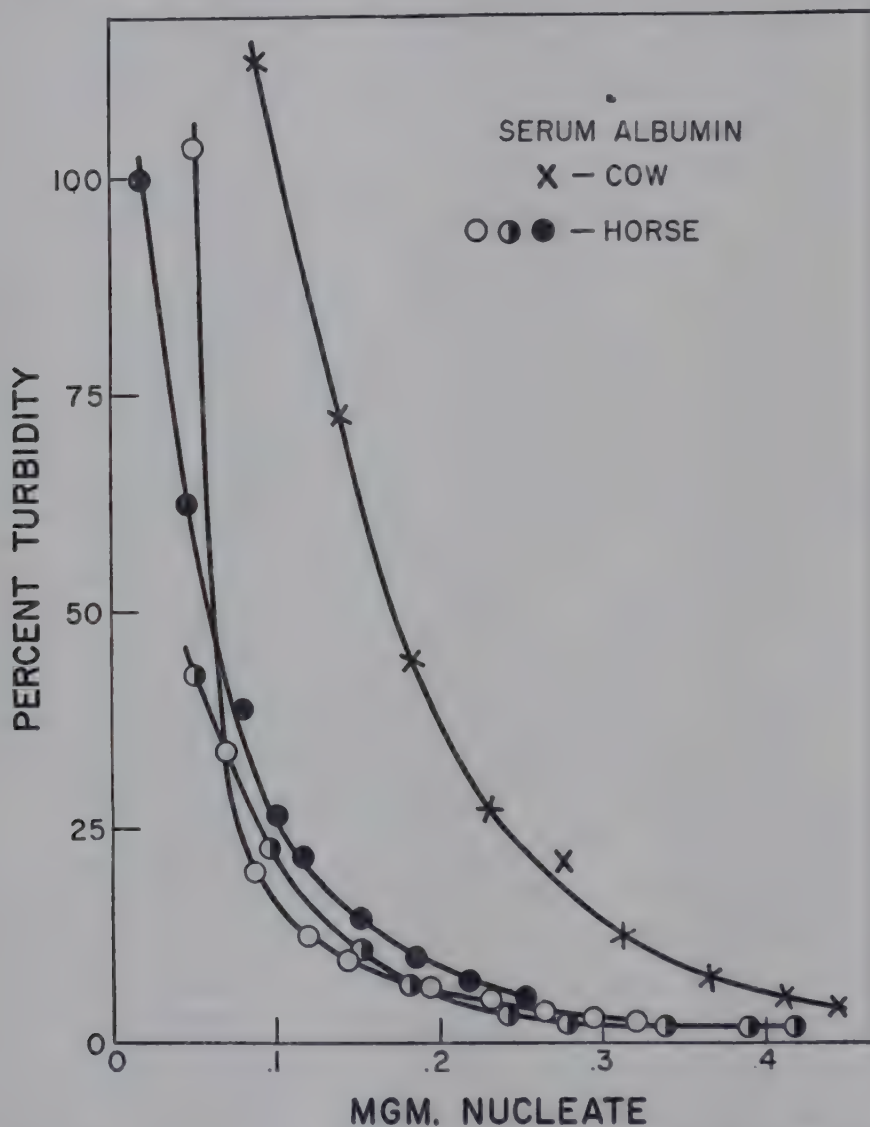


FIG. 1. Protection against heat coagulation of horse and serum albumin by sodium thymus nucleate. Test mixtures composed of 2 cc. of 2.3 per cent albumin solution, 1.9 to 1.0 cc. of water, and 0.1 to 1.0 cc. of nucleate solutions, so that the total volume was 4.0 cc. The control mixture (100 per cent turbidity) was composed of 2 cc. of albumin solution plus 2 cc. of water. X, bovine serum albumin initially with 0.046 per cent nucleate solution; O, O, ●, horse serum albumin initially with 0.046, 0.031, and 0.023 per cent nucleate solutions respectively.

duced a rapid coagulation of protein. We have subsequently noted similar phenomena in the case of the albumins reported herein.²

² Aqueous extracts of liver tumors (about 1 gm. of tissue to 5 cc. of water) do not coagulate at 98° (22). This difference in behavior from that of extracts of normal liver at the same protein concentration was interpreted as possibly being due to the

The test mixtures consisted of 2 cc. of the bovine, horse, and chicken albumins described above, 1 cc. of water, and 1 cc. of 0.046 per cent thymus nucleate. The control mixtures consisted of 2 cc. of the albumin solutions plus 2 cc. of water. Test and control mixtures were heated for 10 minutes in a boiling water bath and then cooled. The test mixtures

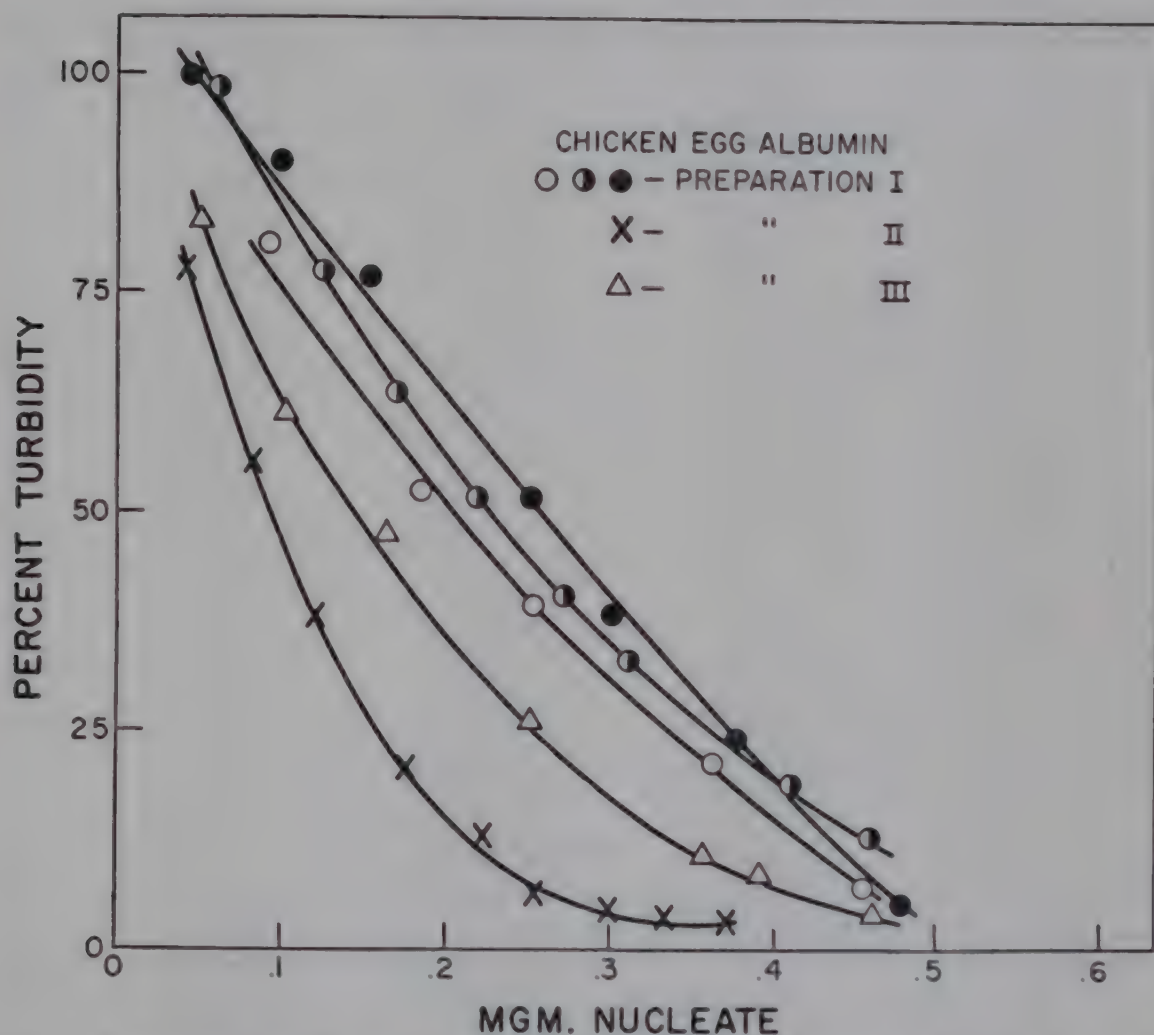


FIG. 2. Protection against heat coagulation of chicken egg albumin by sodium thymus nucleate. The mixtures are the same as in Fig. 1, except that albumin Preparation II was 1.6 per cent. ○, ●, ◐, Preparation I initially with 0.092, 0.058, and 0.046 per cent nucleate solutions respectively; ×, Preparation II initially with 0.04 per cent nucleate; △, Preparation III initially with 0.058 per cent nucleate.

were nearly water-clear. To test and control mixtures were added 0.2 cc. of water containing 1 mg. of desoxyribonuclease (donated by Dr. M.

higher nucleic acid content of the tumor as compared with the normal tissue of origin (22). In partial accord with this suggestion, we have noted that heated (and subsequently cooled) clear extracts of liver tumor coagulated rapidly when digested at 37° with desoxyribonuclease-Mg⁺⁺. No appreciable coagulation of such extracts was noted in the presence of either desoxyribonuclease or magnesium acetate alone.

McCarty) and 0.2 cc. of water containing 0.9 mg. of magnesium acetate. The digests were incubated at 37° and were measured nephelometrically every 15 minutes. In the case of each of the test albumin mixtures an increasing turbidity was observed with the time of incubation. The data for egg albumin are shown in Fig. 3.

The turbidity increased to nearly 50 per cent of the control and then leveled off. Similar curves, although of a somewhat more irregular character, were obtained with horse and bovine serum albumin test mixtures. Heated test mixtures of the albumin with nucleate treated only with

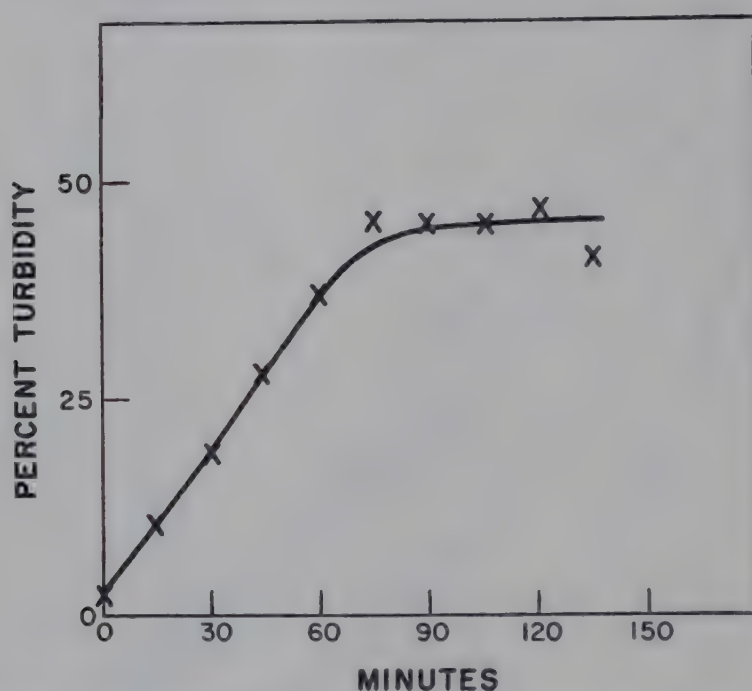


FIG. 3. Increase in turbidity of the protected system of egg albumin-thymus nucleate when incubated with desoxyribonuclease. Digest composed of 2 cc. of 2.3 per cent egg albumin, 1 cc. of water, and 1 cc. of 0.046 per cent thymus nucleate which had been heated at 100° for 15 minutes, cooled to 37°, and treated with 1 mg. of desoxyribonuclease in 0.2 cc. of water and 0.9 mg. of magnesium acetate in 0.2 cc. of water. The comparison mixture (100 per cent turbidity) was the same as above, except that 1 cc. of water substituted for the thymus nucleate solution.

desoxyribonuclease alone, or with magnesium ions alone, gave no appreciable turbidity on incubation (1). Unheated mixtures of the albumins treated with both desoxyribonuclease and magnesium ions yielded no turbidity on incubation at 37°. The pH of both control and test solutions was the same; namely, 6.4.

DISCUSSION

Several salts of the lower fatty acids (2) and of the halogenated acetates (5) have been found to increase the thermal stability of the albumins. It is possible that this property may be related to the well known ability of the albumins to bind anions, such as methyl orange (6-8), sodium do-

decyl sulfate (9-11), and thiocyanate (12). Recent work by Klotz and Urquhart (8) has shown that this property may be a function of the number and distribution of cationic groups in the protein. Thymus nucleate, unlike many of these agents, does not prevent denaturation on heating, but only coagulation.

Electrophoretic studies by Stenhagen and Teorell (13) and by Longworth and MacInnes (14) have suggested, respectively, that some combination occurs between serum albumin and thymus nucleate, and between egg albumin and yeast nucleate. Complex formation between egg albumin and thymus nucleate has also been noted by Björnesjö and Teorell (15). However, there is a considerable difference in the effect on the heat stability of the albumins by the two types of nucleate, for, whereas thymus nucleate protects almost completely against coagulation in relatively small amounts, yeast nucleate has no apparent effect even at relatively high concentrations (1). It is therefore possible that, although the protective effect of thymus nucleate on the heat coagulation of the albumins may be basically due to some form of combination prior to the heating, it is also probable that the protective effect may be due to some additional property of the thymus nucleate. Not all organic anions which bind with proteins protect against the heat coagulation of the latter, or at least protect in equal degree. Thymus nucleate is unique in acting at such low concentrations. Its molecular weight has been reported by several investigators to vary from 500,000 to 2,000,000, depending upon the preparation (15-17). Accepting tentatively the lower figure and a ratio of nearly complete protection against heat coagulation at 98° of 150 mg. of albumin to 1 mg. of nucleate, it would appear that 1 mole of nucleate is sufficient to protect something like 2000 moles of albumin. These figures are not to be taken too seriously, for the molecular weight of such large molecules is at least partly an abstraction, but they suggest the magnitude of the effect produced.

That the albumins actually are denatured in the presence of the nucleate by the heating is shown by the digestion experiments with desoxyribonuclease-Mg⁺⁺ (Fig. 3) (1). The failure of the protein in these digests to coagulate fully may be due to the presence of the split products of the nucleate which might still retain some protective action.³ The products yielded by the action of desoxyribonuclease-Mg⁺⁺ on thymus nucleate

³ It is also possible that, in the presence of thymus nucleate, the albumin is not as completely denatured by heat as in the absence of the nucleate. As shown by Rice *et al.*, addition of sodium caprylate or other similar stabilizing agents apparently prevented those denaturing effects of heat and of urea on serum albumin which ordinarily lead to increased susceptibility of the albumin to papain digestion (23). On the other hand, tissue extracts which had been heated in the presence of thymus nucleate failed to reveal any survival of enzymatic activity, indicating nearly complete denaturation of those tissue enzyme proteins studied (1).

are not yet known. Such products show a lower viscosity (18) and a higher acid solubility (19) than the parent nucleate, and, unlike the latter, are dialyzable through cellophane (19). A decrease in the viscosity of the nucleate solutions does not in itself result in a loss of protection against heat coagulation of albumin, for nucleate solutions which had been irradiated with ultraviolet light at 2537 Å, until the viscosity was reduced to barely more than that of water, still protected as fully as the original solutions (1). The thymus nucleate solutions used in the present studies were so dilute that their viscosity was only slightly greater than that of water. It would appear that the asymmetric molecular character of thymus nucleate was not necessarily concerned with the protective phenomenon, but that some more intimate details of molecular structure in relation to the albumin molecules were concerned. That some mutual interaction between thymus nucleate and protein must occur, whereby the molecular configuration of the nucleate is in turn affected by the protein, is seen in the marked decrease in structural viscosity and streaming birefringence of thymus nucleate solutions when protein solutions at the same pH are added (20). This mutual interaction is inhibited by neutral salts.

Further investigations will be concerned with the nature of the interaction between protein, nucleate, and salt, a phenomenon which Miescher many years ago considered to lie at the very basis of nuclear behavior (21).

Note on Electrophoresis and Sedimentation Studies of Heated Albumin Nucleate Mixtures

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For the purpose of electrophoretic and ultracentrifugal studies, ovalbumin solutions adjusted to pH 6.8 and dialyzed against water were mixed with dialyzed thymus nucleate solutions in weight ratios of albumin to nucleate varying from 50 to 400. The egg albumin concentration was maintained at 1 per cent in the mixture, which was heated at 98°.

Electrophoresis in 0.1 ionic strength sodium diethyl barbiturate buffer at pH 8.6 indicated slight elevation of the ovalbumin mobility, and thymus nucleate was detected even at an albumin to nucleate weight ratio of 200.

Ultracentrifugal studies indicated independence of the patterns obtained on the weight ratio of albumin to nucleate, the time of heating, and the rate of sedimentation. However, the patterns varied markedly, depending on whether the heated mixture was sedimented in water, 0.1 ionic strength sodium diethyl barbiturate, or 0.15 ionic strength sodium chloride, and the effects of the salt medium were reversible. It is of

interest that in sodium diethyl barbiturate most of the material sedimented with a specific sedimentation rate of 14 svedberg units at 30°. Since this is of the same order of magnitude as the sedimentation rate of free nucleate, the soluble heated egg albumin cannot be combined with nucleate as a single sedimenting unit. If this were the case, the molecular weight of the resulting complex would have to be of the order of 50 million to account for all egg albumin molecules present in a mixture of albumin to nucleate weight ratio of 100.

Since the nucleate molecular weight is of the order of 500,000, such huge complex molecules could sediment only at the observed rate if they were linear, which would imply solutions of extreme viscosity. Measurements indicated no appreciable viscosity elevation after heating, however. The interesting conclusion is that partially aggregated egg albumin remains soluble in the presence of protecting thymus nucleate without any tight chemical combination with the nucleate.

SUMMARY

1. Aqueous, salt-free solutions of crystalline preparations of horse and bovine serum albumin and of chicken egg albumin were mixed with varying concentrations of salt-free solutions of sodium thymus nucleate and heated at 98°.

2. At ratios of approximately 1 mg. of nucleate to 100 to 200 mg. of albumin, practically complete protection against heat coagulation was achieved. With decreasing amounts of nucleate, the degree of coagulation of the heated protein was nearly linear.

3. Mixtures of albumin with sufficient nucleate to confer complete protection against coagulation when heated, cooled, and treated with desoxyribonuclease and magnesium ions became gradually coagulated as the protecting nucleate was digested.

BIBLIOGRAPHY

1. Carter, C. E., and Greenstein, J. P., *J. Nat. Cancer Inst.*, **6**, 219 (1946).
2. Ballou, G. A., Boyer, P. D., Luck, J. M., and Lum, F. G., *J. Biol. Chem.*, **153**, 589 (1944).
3. Hammarsten, E., *Biochem. Z.*, **144**, 383 (1924).
4. Taylor, B., Greenstein, J. P., and Hollaender, A., *Arch. Biochem.*, **16**, 19 (1948).
5. Huggins, C., Miller, G. M., and Jensen, E. V., *Cancer Res.*, **9**, 177 (1949).
6. Klotz, J. M., and Walker, F. M., *J. Phys. and Colloid Chem.*, **51**, 666 (1947).
7. Davis, B. D., *J. Clin. Invest.*, **22**, 753 (1943).
8. Klotz, I. M., and Urquhart, J. M., *J. Am. Chem. Soc.*, **71**, 1597 (1949).
9. Neurath, H., *J. Am. Chem. Soc.*, **61**, 1841 (1939).
10. Miller, G. L., and Andersson, K. J. I., *J. Biol. Chem.*, **144**, 475 (1942).
11. Bull, H. B., *J. Am. Chem. Soc.*, **68**, 747 (1946).
12. Scatchard, G., and Black, E. S., *J. Phys. and Colloid Chem.*, **53**, 88 (1949).

13. Stenhagen, E., and Teorell, T., *Tr. Faraday Soc.*, **35**, 743 (1939).
14. Longworth, L. G., and MacInnes, D. A., *J. Gen. Physiol.*, **25**, 507 (1942).
15. Björnesjö, K. B., and Teorell, T., *Ark. Kemi, Mineral. o. Geol.*, **19 A**, No. 34 (1945).
16. Greenstein, J. P., *Advances in Protein Chem.*, **1**, 210 (1944).
17. Kahler, H., *J. Phys. and Colloid Chem.*, **52**, 676 (1948).
18. McCarty, M., *J. Gen. Physiol.*, **29**, 123 (1946).
19. Carter, C. E., and Greenstein, J. P., *J. Nat. Cancer Inst.*, **7**, 29 (1946).
20. Greenstein, J. P., and Jenrette, W. V., *Cold Spring Harbor Symposia Quant. Biol.*, **9**, 236 (1941).
21. Miescher, F., *Die Histochemischen und physiologischen Arbeiten*, Leipzig, **2** (1897).
22. Griffin, A. C., and Baumann, C. A., *Cancer Res.*, **8**, 135 (1948).
23. Rice, R. G., Ballou, G. A., Boyer, P. D., Luck, J. M., and Lum, F. G., *J. Biol. Chem.*, **158**, 609 (1945).

ENZYMATIC HYDROLYSIS OF N-ACYLATED AMINO ACIDS

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The enzymatic susceptibility of certain *N*-acylated L-amino acids, together with the complete resistance of their optical enantiomorphs, has been employed as the basis of a resolution procedure for the separation of the isomers of racemic amino acids (1-4). Both *N*-acetyl and *N*-chloroacetyl derivatives were frequently employed in this procedure, and it was noted that the rates of hydrolysis of the latter were invariably greater than those of the former.

In order to test the enzymatic susceptibility of other types of *N*-acyl substituents, a variety of such derivatives of alanine and of glycine has been prepared. Special attention has been given to the racemic chloropropionyl radical, for, with this substituent, the problem arises as to whether both optical isomers are enzymatically susceptible. The substrates were incubated with preparations of hog kidney.

Of the formyl, acetyl, chloroacetyl, propionyl, *dl*-chloropropionyl, and benzoyl derivatives of alanine, the most susceptible is the chloroacetyl, followed by the acetyl or propionyl, formyl, *dl*-chloropropionyl, and benzoyl. About the same order holds for the corresponding derivatives of glycine. Both optical isomers of *dl*-chloropropionyl-L-alanine are hydrolyzed by hog kidney enzyme preparations, whereas *dl*-chloropropionyl-D-alanine, like all *N*-acylated D-amino acids, is completely resistant. It is interesting to note that, although the chloroacetyl are more susceptible than the acetyl derivatives, the chloropropionyl derivatives are considerably less susceptible than the corresponding propionyl derivatives. The same relative order of susceptibility of chloroacetyl > acetyl > chloropropionyl holds also for the derivatives of dehydroalanine with kidney (5) and of cystine with liver preparations (6). The benzoyl derivatives of the amino acids have apparently a very low order of susceptibility to enzymatic hydrolysis.

EXPERIMENTAL

Formyl-DL-alanine was prepared by heating racemic alanine with anhydrous formic acid, and crystallized in the form of needles from acetone;

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m.p. 150°; N calculated 11.9, found 11.9. The preparation of acetyl-DL-alanine (1) and chloroacetyl-DL-alanine (7) has been described. Benzoyl-DL-alanine (m.p. 162°; N calculated 7.2, found 7.2) was synthesized in the usual manner from benzoyl chloride and alanine in alkaline solution. Propionyl-DL-alanine was prepared by the interaction of propionic anhydride and alanine in slightly alkaline solution; glistening prisms from acetone; m.p. 102°; N calculated 9.6, found 9.6.

dl-Chloropropionyl-L-alanine was prepared by the interaction of *dl*-chloropropionyl chloride (6) with L-alanine ($[\alpha]_D = +14.4^\circ$) in alkaline solution. After acidification with HCl, the reaction mixture was extracted several times with ethyl acetate, and the extracts combined, dried, and evaporated to a small volume. The product crystallized readily, and was recrystallized from acetone in the form of glistening prisms; m.p. 157°; N calculated 7.8, found 7.8. $[\alpha]_D = -20.9^\circ$ for a 4.00 per cent solution in absolute alcohol. *dl*-Chloropropionyl-D-alanine was prepared in the same manner from D-alanine ($[\alpha]_D = 14.4^\circ$); m.p. 157°, N calculated 7.8, found 7.8; $[\alpha]_D = +20.8^\circ$ for a 4.00 per cent solution in absolute alcohol.¹

The preparation of acetylglycine (1) and chloroacetylglycine (1) has been described. Propionylglycine was prepared by the interaction of propionic anhydride and glycine in alkaline solution; m.p. 128°, N calculated 10.7, found 10.5. *dl*-Chloropropionylglycine was synthesized with *dl*-chloropropionyl chloride and glycine in the same fashion as the corresponding alanine compound; m.p. 104°; N calculated 8.7, found 8.3.

The substrates were made up in 0.050 M solution and neutralized with dilute sodium hydroxide to a pH of 7.0. The digests consisted of 1 cc. of hog kidney aqueous extract at appropriate dilutions, 1 cc. of 0.06 M phosphate buffer at pH 7.1, and 1 cc. of either substrate solution or water. The hydrolysis was followed by ninhydrin-CO₂ manometric determinations, and the rate calculated from the initial reaction up to 20 to 30 per cent hydrolysis. The temperature was 38°. No spontaneous hydrolysis of any of the substrates was observed. The data are given in Table I, and refer only to the hydrolysis of the L isomer of alanine. Previous studies have shown that the presence of the resistant acylated D isomer does not affect the rate of hydrolysis of the acylated L form (8, 9).

In order to see whether both isomers of *dl*-chloropropionyl-L-alanine

¹*dl*-Bromopropionyl-L-alanine (m.p. 173°; N calculated 6.3, found 6.2, $[\alpha]_D = -20.9^\circ$ for a 4.00 per cent solution in alcohol) and *dl*-bromopropionyl-D-alanine (m.p. 173°; N found 6.3, $[\alpha]_D = +20.9^\circ$ for a 4.00 per cent solution in alcohol) were prepared by the interaction of α -bromopropionyl bromide with the respective alanes in alkaline solution. Acidification of the reaction mixture with concentrated HCl led to the crystallization of the expected compounds. On recrystallization from acetone, large prisms were obtained. Attempts to aminate these compounds in aqueous ammonia or in ammoniacal methanol were unsuccessful.

were hydrolyzed, digests containing a purified preparation of hog kidney (1) were employed. For a similar study with *dl*-chloropropionyl-D-alanine, a crude concentrated hog kidney extract was employed. Otherwise, the

TABLE I
Rates of Hydrolysis of N-Acylated Derivatives of Alanine and of Glycine

Substrate	Substrate hydrolyzed per hr. per mg. N
	μM
Formyl-DL-alanine.....	22
Acetyl-DL-alanine.....	203
Chloroacetyl-DL-alanine.....	800
Propionyl-DL-alanine.....	220
<i>dl</i> -Chloropropionyl-L-alanine.....	10
<i>dl</i> -Chloropropionyl-D-alanine.....	0
Benzoyl-DL-alanine.....	2
Acetylglycine.....	50
Chloroacetylglycine.....	133
Propionylglycine.....	20
<i>dl</i> -Chloropropionylglycine.....	1
Benzoylglycine.....	0.6

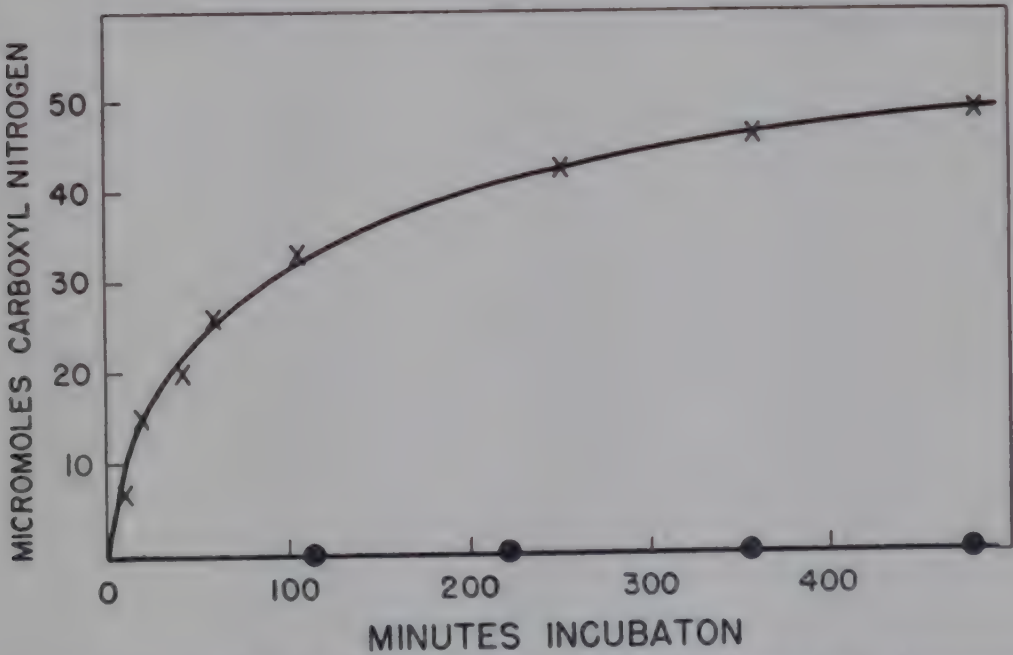


FIG. 1. Hydrolysis of *dl*-chloropropionyl-L-alanine (X) and of *dl*-chloropropionyl-D-alanine (●) by hog kidney preparations at pH 7.0 in phosphate buffer and at 38°. Substrate concentrations 0.050 M.

digests were the same as described above (Table I). The data are given in Fig. 1.

Although both diastereoisomeric forms of *dl*-chloropropionyl-L-alanine

are completely hydrolyzed over the time interval studied, one of them appears to be hydrolyzed at a much faster rate than the other.

SUMMARY

1. The enzymatic hydrolysis by hog kidney preparations of a number of *N*-acylated derivatives of alanine and of glycine was studied. The rate depends on the nature of the acyl radical, the order being in descending fashion for alanine and for glycine: chloroacetyl, acetyl or propionyl, formyl, *dl*-chloropropionyl, and benzoyl.

2. Both optical isomers of *dl*-chloropropionyl-L-alanine were enzymatically cleaved by hog kidney, whereas *dl*-chloropropionyl-D-alanine was completely resistant.

BIBLIOGRAPHY

1. Fodor, P. J., Price, V. E., and Greenstein, J. P., *J. Biol. Chem.*, **178**, 503 (1949).
2. Price, V. E., Gilbert, J. B., and Greenstein, J. P., *J. Biol. Chem.*, **179**, 1169 (1949).
3. Gilbert, J. B., Price, V. E., and Greenstein, J. P., *J. Biol. Chem.*, **180**, 473 (1949).
4. Greenstein, J. P., Gilbert, J. B., and Fodor, P. J., *J. Biol. Chem.*, **182**, 451 (1950).
5. Price, V. E., and Greenstein, J. P., *J. Biol. Chem.* **171**, 477 (1947).
6. Greenstein, J. P., and Leuthardt, F. M., *Arch. Biochem.*, **18**, 377 (1948).
7. Fischer, E., *Ber. chem. Ges.*, **36**, 2982 (1903).
8. Fodor, P. J., Price, V. E., and Greenstein, J. P., *J. Biol. Chem.*, **180**, 193 (1949).
9. Fodor, P. J., and Greenstein, J. P., *J. Biol. Chem.*, **181**, 549 (1949).

THE TOXICITY OF VITAMIN B₆, 4-DESOXYPYRIDOXINE, AND 4-METHOXYMETHYLPYRIDOXINE, ALONE AND IN COMBINATION, TO THE CHICK EMBRYO*

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Ott (1, 2) has shown that desoxypyridoxine (2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine) and methoxypyridoxine (2-methyl-3-hydroxy-4-methoxymethyl-5-hydroxymethylpyridine), analogues of pyridoxine, act as pyridoxine antagonists in the chick. Because of our interest in the effects of metabolic antagonists on the development of the chick embryo (3), and on the behavior of mouse tumors growing on the chorioallantoic membrane of the chick embryo (4), these substances, and various forms of vitamin B₆, were studied in the fertile chick egg.

Materials and Methods

Fertile white Leghorn eggs were obtained from a commercial source. The eggs were incubated at 38° and 75 per cent relative humidity. Pyridoxine hydrochloride, pyridoxamine dihydrochloride, pyridoxal hydrochloride and its phosphate, and methoxy- and desoxypyridoxine hydrochloride were used in this study. These chemicals were dissolved in saline just before use, and introduced into the yolk sac through a hole drilled in the blunt end of the egg. The opening was then sealed with a drop of paraffin. The volume of solution injected into each egg ranged between 0.05 to 0.2 cc. Eggs varying from 0 to 13 days of incubation were used. Following injection, the eggs were candled daily, and the dead embryos were weighed and examined for gross developmental abnormalities. Observations were usually made for 10 days after injection, and embryos surviving beyond this period were often sacrificed. More than 1850 embryos were used in this study. The number of embryos used at each dosage of the injected drugs varied from six to twenty, and at critical dosages several experiments were run. It was not feasible to make accurate LD₅₀ determinations for each compound, or combinations of compounds, in embryos of various ages, and only approximate LD₅₀ values, sufficient to characterize

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each situation, were estimated by inspecting a graph of the pertinent data. In studying the protective value of various forms of vitamin B₆ against the antagonists, the ratio permitting 50 per cent survival of embryos was approximated.

Results

Toxicity of Vitamin B₆ and Related Compounds to Chick Embryo

The approximate LD₅₀ values of the compounds tested in the chick embryo after various periods of incubation are summarized in Table I.

Cravens and Snell (5) tested some of these compounds in the 6 day embryo, and our values are in general accord. They found that pyridoxine was toxic between 5 and 10 mg. per egg, pyridoxal hydrochloride at 5 mg., desoxypyridoxine at 5 mg., and pyridoxamine was non-toxic at 10 mg. per egg, the highest dose tested. When injected at 0 days, desoxypyridoxine produced 100 per cent deaths at doses of 0.5 to 1.0 mg. per egg, a somewhat smaller dose than proved toxic in our experiments.

The most interesting toxicity data were obtained with methoxypyridoxine, and this compound was consequently studied in considerable detail. Only 0.04 mg. per egg of methoxypyridoxine was required to produce 50 per cent mortality at 0 days, but as the embryo became older, this amount became progressively greater, so that at 13 days it was 3.5 mg. per egg. This rise in the LD₅₀ dose roughly paralleled the increase in mass of the chick embryo. Embryonic deaths usually occurred within 2 to 5 days after the injection of the drug; the older embryos receiving a fatal dose tended to survive longer than the younger ones.

Embryonic abnormalities were not consistently found with these agents, but occasionally gross malformations were seen in embryos surviving for long periods, particularly following the injection of desoxypyridoxine, pyridoxal hydrochloride, or pyridoxal phosphate. In embryos intoxicated by methoxypyridoxine, apparently normal growth and development proceeded for the several days before they succumbed. The mechanism whereby methoxypyridoxine caused the death of the embryo is not known, but it did not appear to interfere with growth.

Counteraction of Effects of Antagonists by Vitamin B₆

Pyridoxine, pyridoxamine, pyridoxal hydrochloride, and pyridoxal phosphate gave clear cut protection to the 4 day chick embryo against methoxypyridoxine. The studies on the protective activity of each form of vitamin B₆ are described separately, and the results are summarized in Fig. 1.

Pyridoxine—The 4 day embryo was used in most of the experiments, but a few studies were carried out with 0 and 13 day embryos. In the 4

day embryo, 0.07 mg. per egg of pyridoxine protected 50 per cent of the embryos against 0.5 mg. per egg of methoxypyridoxine ($3 \times \text{LD}_{50}$). As the dose of methoxypyridoxine was raised, a much smaller increase in pyridoxine was necessary to provide protection; at the highest dose of methoxypyridoxine tested, 40 mg. per egg ($240 \times \text{LD}_{50}$), 0.5 mg. of

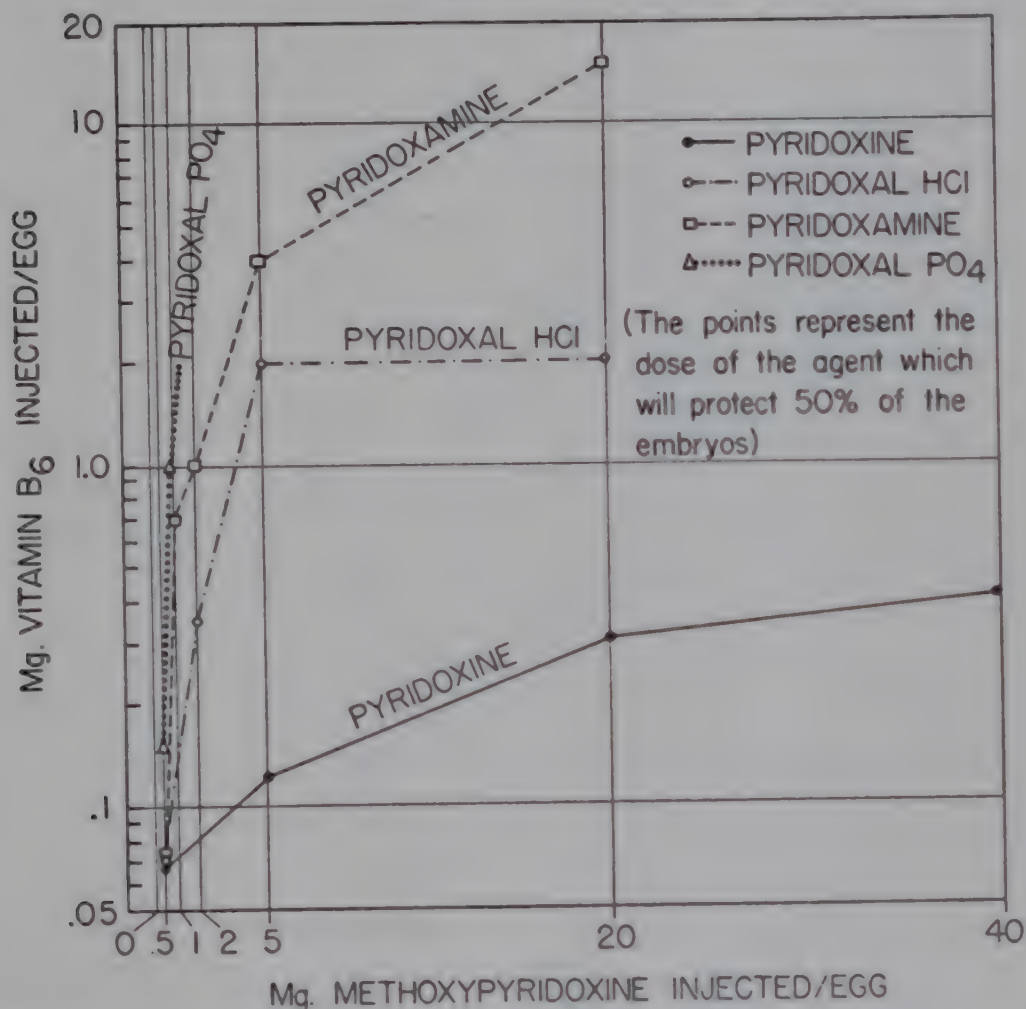


FIG. 1. The protective activity of various amounts of pyridoxine, pyridoxal hydrochloride, pyridoxamine, and pyridoxal phosphate against a range of doses of methoxypyridoxine in the 4 day chick embryo. The toxicity estimates are based on embryonic deaths occurring during the period of 2 to 7 days after injection. The estimated LD_{50} , as charted, is the amount of the vitamin B_6 compound protecting 50 per cent of the embryos against a given dose of methoxypyridoxine. Ten to 90 embryos (average of forty) were used to obtain each point on the chart. The abscissa is plotted on a logarithmic scale.

pyridoxine protected 70 per cent of the embryos. It is of interest that, in the presence of pyridoxine, methoxypyridoxine has a lower intrinsic toxicity (>40 mg. per egg) than pyridoxine, pyridoxal hydrochloride, or pyridoxal phosphate.

Scattered observations on embryos of different ages suggest that the protective ratio of pyridoxine to methoxypyridoxine is more closely re-

lated to the LD₅₀ dose of methoxypyridoxine, rather than to the absolute dose of the drug. For example, 0.2 mg. per egg of pyridoxine failed to protect 0 and 4 day embryos against 5 and 20 mg. per egg, respectively ($120 \times \text{LD}_{50}$) of methoxypyridoxine, whereas it protected 50 per cent of 4 day embryos against 5 mg. ($30 \times \text{LD}_{50}$), and 0.1 mg. of pyridoxine protected 50 per cent of 13 day embryos against 20 mg. ($6 \times \text{LD}_{50}$) of methoxypyridoxine.

Pyridoxine, injected several days prior to methoxypyridoxine, was still capable of protecting against the antagonist. Thus, when 0.5 mg. of pyridoxine was injected into 2 day embryos, it protected them against the injection of 5 mg. of methoxypyridoxine made 2 days later. Similarly, the injection of 1 mg. of pyridoxine at 4 days of incubation protected against 20 mg. of methoxypyridoxine injected at 10 days. Thus pyridoxine remains active for at least 6 days after injection into the egg.

The ability of pyridoxine to reverse the toxicity of methoxypyridoxine was tested in the 4 day embryo. When 5 mg. of methoxypyridoxine were followed 1, 2, 4, 8, 24, or 48 hours later with 0.5 mg. of pyridoxine, those embryos receiving pyridoxine within 24 hours showed appreciable protection, whereas all embryos injected at 48 hours died. The effects of a large dose of methoxypyridoxine ($30 \times \text{LD}_{50}$) thus may be reversed by pyridoxine within 24 hours, but not at 48 hours. The great majority of deaths in the 4 day embryos treated with methoxypyridoxine occurred between 48 and 72 hours, so the failure of pyridoxine to reverse the toxicity of methoxypyridoxine at 48 hours is not surprising.

Pyridoxal Hydrochloride and Pyridoxamine—These compounds appeared to be as active as pyridoxine in counteracting the toxic effects of 0.5 mg. per egg of methoxypyridoxine. As the dosage of methoxypyridoxine was increased, the amounts of these agents necessary to provide protection rose promptly to higher levels, and pyridoxal hydrochloride showed considerably more protective activity than pyridoxamine (Fig. 1).

Pyridoxal Phosphate—This agent was found to have weak protective activity against methoxypyridoxine. It provided protection against 0.5 mg. per egg of methoxypyridoxine, but when the dose of the antagonist was increased above 1 mg. per egg, pyridoxal phosphate, given in doses up to its limiting intrinsic toxicity (3 to 5 mg. per egg), failed to protect the embryos.

Desoxypyridoxine—Cravens and Snell (5) found that vitamin B₆ would protect 0 day embryos against desoxypyridoxine, but in the 4 to 6 day embryo vitamin B₆ could not diminish the toxicity of desoxypyridoxine. Similarly, in our experiments with the 4 day embryo, desoxypyridoxine did not show any vitamin B₆ or antivitamin B₆ activity. The LD₅₀ of desoxypyridoxine in the 4 day embryo is 4 mg. per egg. At 5 mg. of

desoxypyridoxine, 5 and 3 mg. of pyridoxine and pyridoxal phosphate, respectively, did not provide any protection. Conversely, 2 mg. of desoxypyridoxine did not protect the chick embryo against 0.5 mg. of methoxypyridoxine.

DISCUSSION

A number of interesting differences between the chemically closely related compounds studied have been brought out by the use of the chick embryo. If the antivitamin B₆ activity of methoxypyridoxine is eliminated by adding pyridoxine, these agents show the following order of toxicity in the 4 day embryo: methoxypyridoxine, not toxic at 40 mg. per egg, the highest level tested, in the presence of a protective amount of pyridoxine; pyridoxamine, not toxic at 20 mg. per egg, the highest level

TABLE I

Approximate LD₅₀ Doses of Vitamin B₆ and Related Compounds for Chick Embryos of Different Ages

The values are expressed as mg. per egg.

Age of embryo when injected	Pyridoxine	Pyridoxamine	Pyridoxal	Pyridoxal phosphate	Desoxy- pyridoxine	Methoxy- pyridoxine
<i>days</i>						
0			>3.0		1.0-2.0	0.04
2						0.07
4	12.0	>20.0	5.0	3.0-5.0	4.0	0.17
6						0.25
8						0.80
10						0.90
13	>20.0	>20.0	5.0		12.0	3.50

tested; pyridoxine, approximate LD₅₀, 12 mg. per egg; pyridoxal hydrochloride and pyridoxal phosphate, LD₅₀ range, 3 to 5 mg. per egg; desoxypyridoxine, approximate LD₅₀, 4 mg. per egg.

These limited data, and those contained in Table I, suggest that the toxicity of these agents, in the presence of vitamin B₆, shows relatively little change in the chick embryo from 0 to 13 days of age, particularly as contrasted with the marked decrease in the toxicity of methoxypyridoxine with the increase in the age of unprotected embryos. The mechanisms of the lethal action of these agents, as apparently dissociated from their vitamin B₆ activity of antagonism, present an interesting problem.

The embryo can be protected against the toxic effects of methoxypyridoxine (and this was studied particularly in the 4 day embryo) by vitamin B₆, and considerable differences were found in the protective activity of the different vitamin B₆ compounds. The protective dose of pyridoxine

was shown to increase relatively little with large increases in methoxypyridoxine. Pyridoxal hydrochloride and pyridoxamine showed protective activity approximately equal to that of pyridoxine at $3 \times \text{LD}_{50}$ doses of methoxypyridoxine, but as the dose of methoxypyridoxine was increased, these compounds appeared less active than pyridoxine, and pyridoxamine was less active than pyridoxal hydrochloride. Pyridoxal phosphate was the least active of the vitamin B₆ compounds tested against $3 \times \text{LD}_{50}$ doses of methoxypyridoxine, and it did not protect against larger doses. On the basis of the relative protective plateaus in dosage that these agents (excluding pyridoxal phosphate) achieve against 5 mg. and higher doses of methoxypyridoxine, their relative protective activities against methoxypyridoxine are estimated as 30 (pyridoxine) to 3 (pyridoxal hydrochloride) to 1 (pyridoxamine) (Fig. 1).

Our results with desoxypyridoxine are in accord with those of Cravens and Snell (5). In their experiments with the 0 day eggs, desoxypyridoxine was lethal at doses of 1.0 mg. per egg. 50 per cent of the embryos was protected against this dose by approximately 0.01 mg., 0.02 mg., and 0.05 mg. of pyridoxine, pyridoxamine, and pyridoxal hydrochloride, respectively. In 4 and 6 day embryos, however, larger amounts of desoxypyridoxine were required to show toxicity, and none of these agents protected against these increased amounts of desoxypyridoxine; they conclude that this toxic action is not due to the antivitamin B₆ activity of desoxypyridoxine. Our data support this view, and it seems probable that desoxypyridoxine can intoxicate the chick embryo by two different mechanisms. The first mechanism is unrelated to its antivitamin B₆ activity, and may be referred to as its intrinsic toxicity. The intrinsic toxicity of desoxypyridoxine rises from about 2 mg. per egg at 0 day to 12 mg. per egg at 13 days, and this toxic activity may be related to those of pyridoxine, pyridoxal hydrochloride, and pyridoxal phosphate (Table I).

The second mechanism by which desoxypyridoxine is toxic is due to its antivitamin B₆ activity. At 0 day, desoxypyridoxine is toxic at 1.0 mg. per egg, and this action can be prevented by vitamin B₆. As the embryo becomes older, a larger amount of antivitamin B₆ activity is necessary to produce embryonic death. For example, the LD_{50} dose of methoxypyridoxine increases from 0.04 mg. per egg at 0 day to 0.17 mg. per egg at 4 days, a 4-fold increase. If it is assumed that the dose of desoxypyridoxine must be increased proportionally in order to produce a lethal antivitamin B₆ effect in the 4 day embryo, the estimated dose of desoxypyridoxine necessary will produce death because of its intrinsic toxicity. Methoxypyridoxine is, thus, far superior to desoxypyridoxine as an antagonist of vitamin B₆ in the chick embryo, since it has a much lower in-

trinsic toxicity, and from the above considerations it is estimated to be at least 25 times more active than desoxypyridoxine as a vitamin B₆ antagonist.

The resistance of the embryo to methoxypyridoxine as it increases in age prompts speculation. This may mean that the embryo, as it grows, is forming vitamin B₆, or, as suggested by Cravens and Snell (5), that the vitamin B₆-dependent systems, as they develop, become resistant to the antagonist. The suggestion, in our data, that the dose of pyridoxine protecting embryos against methoxypyridoxine is more closely related to the LD₅₀ dose of the antagonist at a given age than to the absolute amount of the drug does not clarify this problem. The vitamin B₆ content of the egg must be considered as a factor in these experiments, particularly since Rabinowitz and Snell (6) have reported that each gm. of dried egg contains 0.0056 mg. of pyridoxal hydrochloride, 0.0012 mg. of pyridoxamine, and a negligible amount of pyridoxine. This may be roughly estimated as about 0.08 mg. of vitamin B₆ per whole egg, the significance of which in relation to our observations is not clear.

The mechanism whereby methoxypyridoxine antagonizes vitamin B₆ is unknown. Umbreit and Waddell (7) have studied the antagonistic action of desoxypyridoxine *in vitro*. They conclude that desoxypyridoxine is phosphorylated and competes with pyridoxal phosphate, the active form of vitamin B₆. In the chick embryo, however, the facts that pyridoxine, pyridoxal hydrochloride, pyridoxamine, and pyridoxal phosphate are effective against methoxypyridoxine in a descending order, and that pyridoxine, once it achieves a certain level, will protect against increasing doses of methoxypyridoxine, suggest that pyridoxine is the most active form of vitamin B₆ of those tested.

The exact rôle of vitamin B₆ in the metabolism of birds and mammals has not been demonstrated. It is known, however, to play an important rôle as a coenzyme in transamination, decarboxylation, and possibly in carboxylation reactions, and Bonner and Bonner (8) have stated that, "In its possible rôle as a coenzyme in transamination, in particular, it may occupy a key position in the synthesis... of the proteins of the plant." It is of interest, therefore, that chick embryos treated with lethal doses of methoxypyridoxine continue to differentiate and enlarge for the 2 to 5 day period before death, presumably due to the continued synthesis of protein and the formation of new cells. It appears likely that the vitamin B₆ deficiency induced by methoxypyridoxine in the chick embryo produces death by means of a physiological derangement, not immediately related to the processes of tissue growth, or by the accumulation, in the closed system of the egg, of lethal concentrations of the by-products of a vitamin B₆ deficiency. One of these limiting factors, in producing early embryonic

death, may mask the development of more complete or profound evidences of a vitamin B₆ deficiency. At the moment, it can only be concluded that the effects of methoxypyridoxine in the chick embryo may permit a further exploration of the mechanism of action of vitamin B₆ and supply a new method for assaying compounds for vitamin B₆ activity.

SUMMARY

Methoxypyridoxine is toxic to the chick embryo because of its anti-vitamin B₆ activity. Its toxicity decreases with the age of the embryo, the LD₅₀ increasing from 0.04 mg. per egg at 0 day to 0.17 mg. per egg at 4 days and 3.5 mg. at 13 days. The lethally intoxicated embryos may survive for several days and continue to show grossly normal growth and development. Pyridoxine, pyridoxal hydrochloride, pyridoxamine, and pyridoxal phosphate will protect the chick embryo against methoxypyridoxine, and their activity decreases in the order listed. A relatively constant amount of pyridoxine will protect the embryo against a wide range of concentrations of methoxypyridoxine, whereas, at the other extreme, pyridoxal phosphate protects against only a small amount of methoxypyridoxine.

Desoxypyridoxine is a weak vitamin B₆ antagonist in the 0 day chick embryo, as shown by Cravens and Snell (5). In older embryos its intrinsic toxicity proves lethal at levels which do not apparently produce a severe vitamin B₆ deficiency, as evidenced by the failure of vitamin B₆ to protect the embryo against its action. It is estimated that methoxypyridoxine is at least 25 times more active than desoxypyridoxine as a vitamin B₆ antagonist in the 0 to 4 day chick embryo.

BIBLIOGRAPHY

1. Ott, W. H., *Proc. Soc. Exp. Biol. and Med.*, **61**, 125 (1946).
2. Ott, W. H., *Proc. Soc. Exp. Biol. and Med.*, **66**, 215 (1947).
3. Karnofsky, D. A., Patterson, P. A., and Ridgway, L. P., *Proc. Soc. Exp. Biol. and Med.*, **71**, 447 (1949).
4. Karnofsky, D. A., Burchenal, J. H., Ormsbee, R. A., Cornman, I., and Rhoads, C. P., in Moulton, F. R., *Approaches to tumor chemotherapy*, American Association for the Advancement of Science, Washington, 293 (1947).
5. Cravens, W. W., and Snell, E. E., *Proc. Soc. Exp. Biol. and Med.*, **71**, 73 (1949).
6. Rabinowitz, J. C., and Snell, E. E., *J. Biol. Chem.*, **176**, 1157 (1948).
7. Umbreit, W. W., and Waddell, J. A., *Proc. Soc. Exp. Biol. and Med.*, **70**, 293 (1949).
8. Bonner, J., and Bonner, H., in Harris, R. S., and Thimann, K. V., *Vitamins and hormones*, New York, **6**, 225 (1948).

CHOLESTEROL ESTERASES

II. CHARACTERIZATION OF THE ESTERIFYING CHOLESTEROL ESTERASE OF PANCREATIN*

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The preparation of a substrate mixture and the characterization of the hydrolytic cholesterol esterase system of pancreatin have been reported recently (1). A comparable substrate mixture, suitable for studying the activity of the esterifying cholesterol esterase, has been developed and utilized for characterizing the esterifying system of pancreatin. During the course of the characterization studies, it was found that a stable emulsion of cholesterol could be produced in the absence of bile salts by the general procedure previously described (1). This finding allowed a study of the effect of bile salts on the esterifying system and it was found that bile salts were necessary for the activity of the enzyme. Nedswedski (2) has previously reported that pancreatic lipase catalyzed the esterification of emulsified cholesterol with fatty acids when bile salts were present but not in their absence. Sperry and Stoyanoff (3) found that bile salts had different effects on the cholesterol esterase of human and dog sera. Small amounts inhibited esterification in the serum of both species. Larger amounts, above the level of complete inhibition of esterification, gave no further effect with human serum, but produced hydrolysis of the cholesterol esters with dog serum. These data emphasized the importance of reinvestigating the rôle of bile salts in the hydrolyzing system of pancreatin. Yamamoto *et al.* (1) were unable to obtain a stable emulsion of cholesterol oleate in the absence of bile salts. However, by incorporation of Tween 20 in the substrate mixture, we have obtained stable emulsions of the ester. Under these conditions, it was found that bile salts were also necessary for the activity of the hydrolyzing system. The present report presents the procedure for preparing a substrate mixture suitable for studying the esterifying cholesterol esterase, a characterization of the esterifying system of pancreatin, and experiments on the effect of bile salts on the hydrolyzing system.

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EXPERIMENTAL

Preparation of Substrate Mixture for Studying Esterifying Cholesterol Esterase—A weighed amount of cholesterol was transferred to each of a series of test-tubes (25×200 mm.), previously tested for use with a stainless steel pestle of the Potter-Elvehjem homogenizing apparatus (4), and 1 cc. of ethyl ether was added to each to dissolve the cholesterol. The following were then added to each tube in order: a measured volume of oleic acid, 1 cc. of 10 per cent sodium taurocholate, 10 cc. of 0.154 M potassium phosphate buffer, 1 cc. of 1:1000 merthiolate, and 500 mg. of egg albumin, impalpable powder, soluble. The mixtures were homogenized for 2 minutes and then shaken in a constant temperature bath at 37° for 1 hour. The tubes were removed from the bath and 1 cc. of enzyme solution or substitute added to each. After mixing and removal of zero hour samples, the tubes were returned to the shaker for the duration of the experiment. The total and free cholesterol content of the samples was determined by the method of Schoenheimer and Sperry (5) as modified by Sperry (6). In the experiments described below, the total cholesterol content of the digests was determined when it was necessary to demonstrate the uniformity of the emulsions during incubation. The percentage esterification in all cases was calculated from the data for free cholesterol. In general the total cholesterol content of samples withdrawn after varying periods of incubation did not vary more than the duplicate analyses on the same sample. This result indicated that, within the limits of accuracy of the method used for determining the cholesterol, the digests were uniform in composition throughout the duration of an experiment.

Characterization of Esterifying Cholesterol Esterase of Pancreatin—In some of the experiments the pancreatin, U. S. P., Merck, was suspended in a glycerol-water mixture (1:1) to give a 20 per cent concentration and is designated in Tables I and II as pancreatin solution. In other experiments the pancreatin solution was centrifuged for 10 minutes at 2500 R.P.M., and the supernatant was used as the enzyme source and is designated in Tables II and IV as pancreatin extract. Several procedures for suspending and extracting the pancreatin were tested. The suspensions were shaken for 1 hour at room temperature before centrifugation or other treatment. Centrifuging decreased the activity slightly. The most active preparations were obtained with a glycerol-water mixture (1:1). These experiments are summarized in Table I. There was no esterification for 24 hours when the pancreatin preparations were boiled for 15 minutes or when distilled water or a glycerol-water mixture (1:1) was substituted for the enzyme preparations.

Effect of Changes in Substrate Mixture on Esterification of Cholesterol—Nieft and Deuel (7) reported that the esterifying cholesterol esterase system of rat liver required phosphate and suggested that the esterifying

system involved some intermediary phosphate compound. The data of Experiment 1 in Table II show that the esterifying system of pancreatin was as active in the presence of citrate buffer as of phosphate. The substrate mixtures prepared with citrate buffer were free of phosphate ion when analyzed by the method of Fiske and Subbarow (8). In Experiment 3, Table II, the effect of substituting 1 cc. of 10 per cent Tween 20 for the sodium taurocholate was determined. The results suggested that the bile salt was required for the action of the enzyme. However, it was possible that the Tween 20 was inhibiting the activity of the enzyme.

TABLE I

Esterifying Activity of Pancreatin Solutions Prepared in Various Ways

The digests were prepared as described in the text. pH of digests, 6.6. Substrate, cholesterol, 75 mg.; oleic acid, 164.4 mg. Enzyme, 1 cc. of 20 per cent pancreatin preparations designated in the table.

Experiment No.	Digest No.	Pancreatin preparation	Esterification	
			6 hrs.	24 hrs.
			<i>per cent</i>	<i>per cent</i>
2	1	Control*	0.0	0.0
	6	Glycerol-water	50.4	72.8
	2	Distilled water	35.2	71.5
	3	" " centrifuged	27.7	67.3
	4	" " boiled	0.0	0.0
	5	" " centrifuged, boiled	0.0	0.0
4	1	Glycerol-water "	55.7	73.2
	4	Saline†	46.0	71.5
	5	" centrifuged	40.5	67.4
	6	Control‡	0.0	0.0

* Distilled water.

† 0.9 per cent.

‡ Glycerol-water (1:1).

Preliminary experiments indicated that bile salts could be omitted from the substrate mixture, prepared as outlined above, without affecting the uniformity or stability of the cholesterol emulsion. Duplicate samples removed from a single digest after various periods of incubation had the same total cholesterol content within the limits of error of the analytical method (5). Thus, the substrate mixture prepared without bile salts or Tween 20 fulfilled the requirements for a suitable substrate mixture as discussed previously (1); namely, that the total cholesterol content of the mixture should be uniform and constant during an experiment. Apparently the protein component of the mixture was adequate for stabilization of the emulsion.

Experiments 4 and 6, Table II, were then carried out and they clearly

demonstrated that bile salts were required for the activity of the enzyme. The data of Experiment 4, Table II, indicate that there was a distinct difference between the effects of the 5 per cent sodium taurocholate and the higher concentrations after 24 hours; the 10, 15, and 20 per cent concentrations all gave the same degree of esterification. However, after 6 hours incubation there were small but significant differences in the effects of these concentrations of sodium taurocholate. The results of

TABLE II

Effect of Changes in Substrate Mixture on Esterification of Cholesterol by Cholesterol Esterase of Pancreatin

The digests were prepared as described in the text. pH of digests, 6.6. Enzyme, Experiments 1 and 4, 1 cc. of 20 per cent pancreatin solution; Experiments 3 and 6, 1 cc. of 20 per cent pancreatin extract. Substrate, cholesterol, 75 mg.; oleic acid, 164.4 mg.

Experiment No.	Digest No.	Buffer	Sodium taurocholate	Esterification	
				6 hrs.	24 hrs.
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	1	Phosphate	10	48.5	76.0
	6	Citrate	10	51.3	78.5
3	1	Phosphate	10	55.7	73.2
	2	"	Tween 20*	0.0	0.0
	3	"	" 20	0.0	0.0
4	1	"	0	0.0	0.0
	2	"	5	35.2	67.2
	4	"	10	54.9	76.5
	5	"	15	58.3	75.6
	6	"	20	61.1	74.8
6	1	"	0	0.0	0.0
	2	"	0	0.0	0.0
	3	"	5	27.1	47.5
	4	"	5	27.2	47.5
	5	"	10	44.9	71.4
	6	"	10	47.1	71.0

* 1 cc. of 10 per cent Tween 20.

Experiment 6, Table II, confirm those of Experiment 4 and also demonstrate the close agreement of duplicate digests in the procedure. The total cholesterol contents of the digests in Experiments 4 and 6, which were determined at 0, 6, and 24 hours, showed that the uniformity and stability of the digests were the same for the different levels of bile salts. Thus, it seems that the activating effect of sodium taurocholate in these experiments must be attributed to some other mechanism than a promotion of emulsification.

Inactivation Temperature—The determination of the inactivation temperature was carried out as previously described (1) for the hydrolytic cholesterol esterase. Portions of the 20 per cent pancreatin solution were heated for 15 minutes at the temperatures designated in Table III and then 1 cc. samples were added to the substrate mixtures and incubated as usual. The data show that the esterifying system was inactivated by heating for 15 minutes at 65°.

Determination of Optimum pH for Esterifying Activity—In the determination of the optimum pH it was not practical to prepare digests at regular pH intervals because of the buffering effects of the protein and bile salt. The procedure adopted was to use potassium phosphate buffers (0.154 M) which, when incorporated into the substrate mixture, would give a digest in the range of pH 4 to 9. There were no significant changes in the pH of the digests during incubation. A composite curve from two

TABLE III

Determination of Inactivation Temperature of Esterifying Cholesterol Esterase of Pancreatin

The digests were prepared as described in the text. pH of digests, 6.6. Substrate, cholesterol, 75 mg.; oleic acid, 164.4 mg. Enzyme, 1 cc. of 20 per cent pancreatin extract incubated for 15 minutes at temperatures designated in the second line of the table.

Digest No.....	1	2	3	4	5	6
Temperature, °C.....	38	45	50	55	60	65
Esterification at 6 hrs., %.....	49.3	38.4	26.7	22.1	4.1	0.0
“ “ 24 hrs., %.....	75.6	75.2	68.1	62.2	16.9	0.0

experiments is presented in Fig. 1. The curve shows that there was a sharp optimum in the activity at pH 6.2; the percentage esterification was distinctly greater at pH 6.2 than at 6.0 or 6.4.

Effect of Concentration of Enzyme—A 20 per cent pancreatin extract was diluted with a glycerol-water mixture (1:1) to give concentrations of 2.5, 5, 10, and 15 per cent. The results shown in Fig. 2 were obtained with 75 mg. of cholesterol and an equimolar quantity of oleic acid (54.8 mg.) per digest. During the first 4 hours of incubation, the percentage esterification per unit amount of enzyme decreased as the enzyme concentration was increased. From the 6th to the 24th hours, the rate of esterification appeared to be independent of the amount of enzyme present, as is evident from the curves which are nearly parallel during the period.

Time-Rate Relationship and Effect of Substrate Concentration—Fig. 3 shows the time-rate curves for six concentrations of cholesterol. For each concentration of cholesterol, oleic acid was added in an equimolar amount.

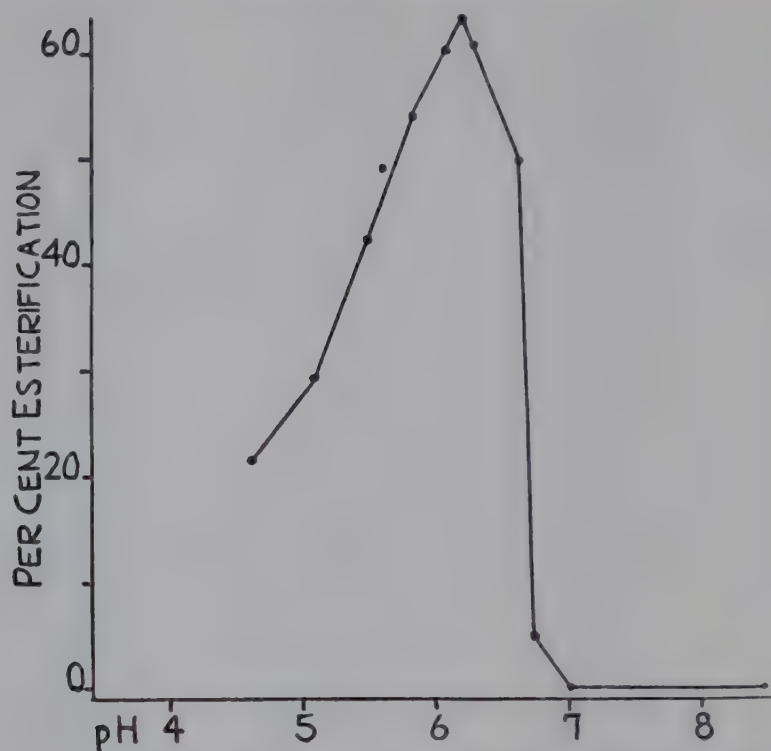


FIG. 1. The influence of pH on the esterification of cholesterol by pancreatin. The digests were prepared as described in the text. Substrate, cholesterol, 75 mg.; oleic acid, 164.4 mg. Incubation time, 6 hours. Enzyme, 1 cc. of 20 per cent pancreatin extract.

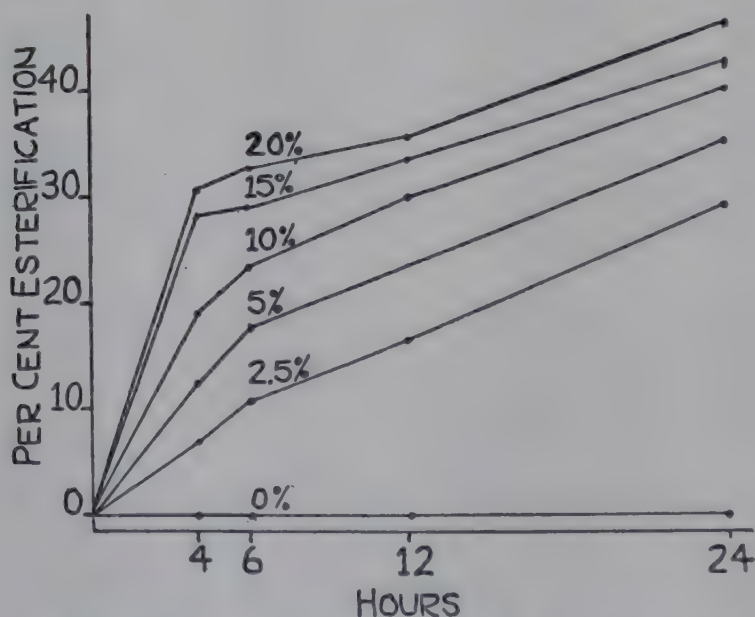


FIG. 2. Effect of concentration of enzyme. The digests were prepared as described in the text. Substrate, cholesterol, 75 mg.; oleic acid, 54.8 mg. pH of digests, 6.2. The concentrations of pancreatin indicated on the curves were prepared by dilution of the 20 per cent pancreatin extract with a glycerol-water mixture (1:1).

Calculation of the kinetic constants indicated that the curves approach those for a second order reaction during the first 6 hours. Further analysis of the data indicated that there was close to a straight line relationship

between the percentage esterification at 6, 12, and 24 hours of incubation and the amount of cholesterol present at zero time. This relationship suggests that at these times the enzyme was not saturated by the substrate.

Effect of Oleic Acid Concentration—Fig. 4 shows the percentage esterification of a constant amount of cholesterol (75 mg.) at five different concentrations of oleic acid. Increasing the oleic acid from 1 to 2 moles per mole of cholesterol increased the esterification approximately 100 per cent. When the oleic acid was 3 and 5 times the equivalent of the cho-

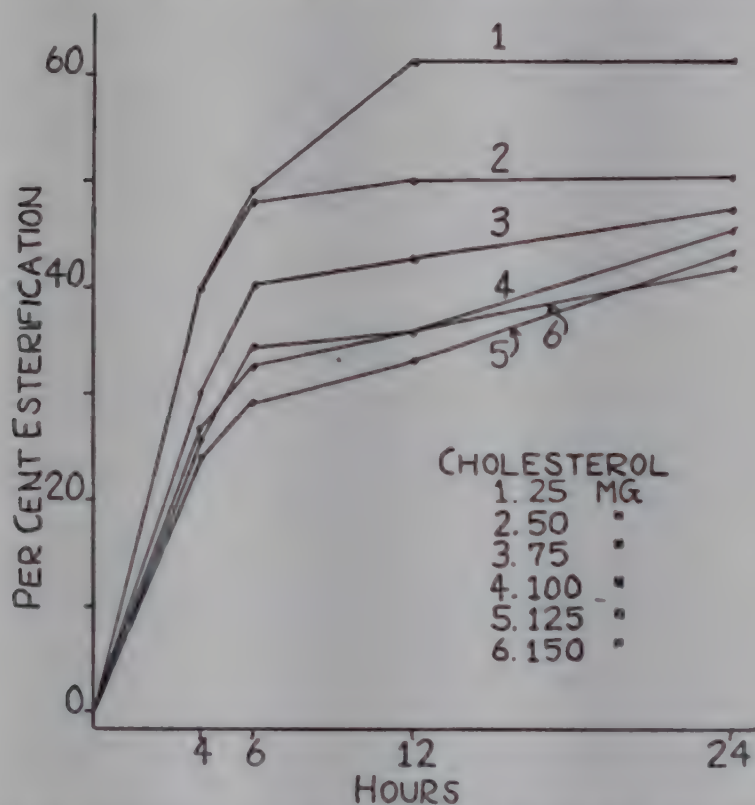


FIG. 3. Time-rate relationship and effect of substrate concentration. The digests were prepared as described in the text. pH of digests, 6.2. Enzyme, 1 cc. of 20 per cent pancreatin extract. Oleic acid, equimolar with cholesterol.

lesterol, there were further small but significant increases in esterification. The absence of esterification in the control digests in which oleic acid (Fig. 4) or enzyme (Fig. 2) was omitted demonstrated that there were no procedural errors in the experiments.

Are Bile Salts Required for Activity of Hydrolytic Cholesterol Esterase of Pancreatin?—The finding that bile salts were required for the activity of the esterifying system emphasized the importance of devising a procedure for producing a stable emulsion of cholesterol oleate which did not involve bile salts. It was found that substitution of Tween 20 for the bile salts and homogenization of the substrate mixture (1) at 37° produced a stable emulsion in the absence of bile salts. Table IV shows two experi-

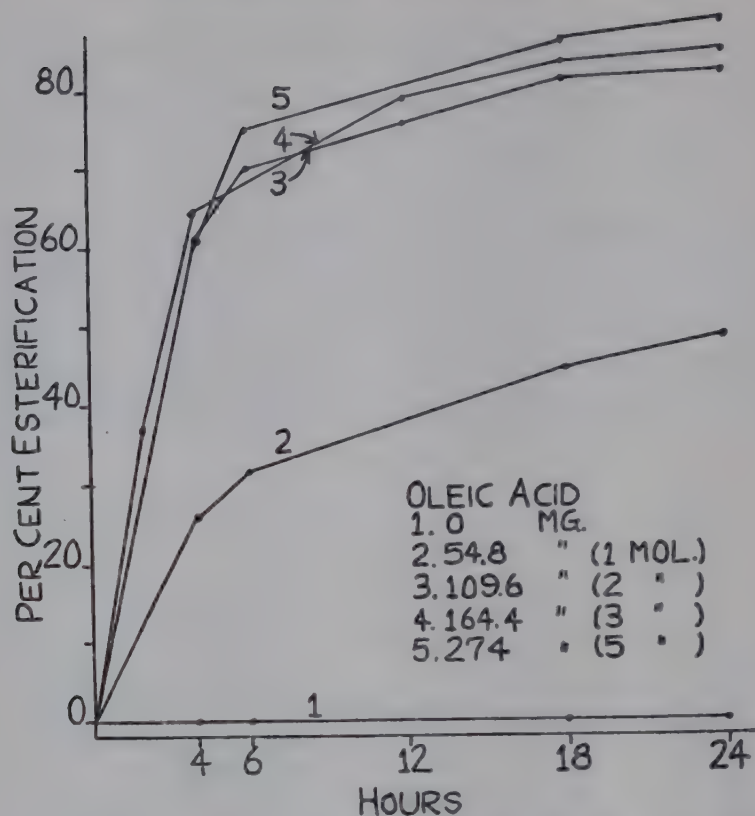


FIG. 4. Effect of oleic acid concentration on esterification of cholesterol by pancreatin. The digests were prepared as described in the text. Cholesterol, 75 mg. pH of digests, 6.2. Enzyme, 1 cc. of 20 per cent pancreatin extract.

TABLE IV

Requirement of Bile Salts for Activity of Hydrolytic Cholesterol Esterase of Pancreatin

The digests were prepared as described in the text. Enzyme, 1 cc. of 20 per cent pancreatin extract.

Experi- ment No.	Digest No.	Choles- terol oleate	pH	Emulsifying agent	Hydrolysis	
					8 hrs.	24 hrs.
					per cent	per cent
1H	1	124.5	6.2	Sodium taurocholate*	16.9	21.1
	2	124.5	6.6	" "	23.0	41.5
	3	124.5	6.2	Tween 20†	0.0	0.0
	4	124.5	6.6	" 20	0.0	0.0
3H	2	75	6.6	Sodium taurocholate		41.7
	3	75	6.6	Tween 20		0.0
	5	75	6.6	Sodium taurocholate + Tween 20		42.5

* 1 cc. of 10 per cent.

† 1 cc. of 10 per cent.

ments representative of several which were performed. All of these demon-
strated that bile salts were also required for the activity of the hydrolytic

system. The data also demonstrate that the failure to hydrolyze the ester in the modified substrate mixture was not due to an inhibiting effect of the Tween 20 on the enzyme. Other data, not reported here, indicated that the percentage hydrolysis observed with a given concentration of the hydrolytic enzyme was related to the concentration of bile salt.

SUMMARY

A substrate mixture, containing cholesterol and oleic acid, and suitable for quantitative studies of the esterifying cholesterol esterase, has been devised. The substrate mixture was used in studying the following characteristics of the esterifying activity of a commercial pancreatin. The enzyme required bile salts and a fatty acid source for activity. It was equally active in the presence of phosphate or citrate buffer. The enzyme was inactivated by heating for 15 minutes at 65°. There was a sharp optimum in the pH-activity curve at 6.2. The time-rate relationships during 24 hours incubation were determined for series of cholesterol, oleic acid, and enzyme concentrations. The enzyme was extracted from pancreatin by a glycerol-water mixture (1:1), water, or saline; the most active extracts were obtained with the glycerol-water mixture.

BIBLIOGRAPHY

1. Yamamoto, R. S., Goldstein, N. P., and Treadwell, C. R., *J. Biol. Chem.*, **180**, 615 (1949).
2. Nedswedski, S. W., *Z. physiol. Chem.*, **236**, 69 (1935).
3. Sperry, W. M., and Stoyanoff, V. A., *J. Biol. Chem.*, **117**, 525 (1937).
4. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
5. Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, **106**, 745 (1934).
6. Sperry, W. M., *Am. J. Clin. Path.*, **8**, Tech. Suppl., **2**, 91 (1938).
7. Nieft, M. L., and Deuel, H. J., Jr., *J. Biol. Chem.*, **177**, 143 (1949).
8. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).

THE INABILITY OF CHOLINE TO TRANSFER A METHYL GROUP DIRECTLY TO HOMOCYSTEINE FOR METHIONINE FORMATION

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WITH THE TECHNICAL ASSISTANCE OF JERARD HURWITZ

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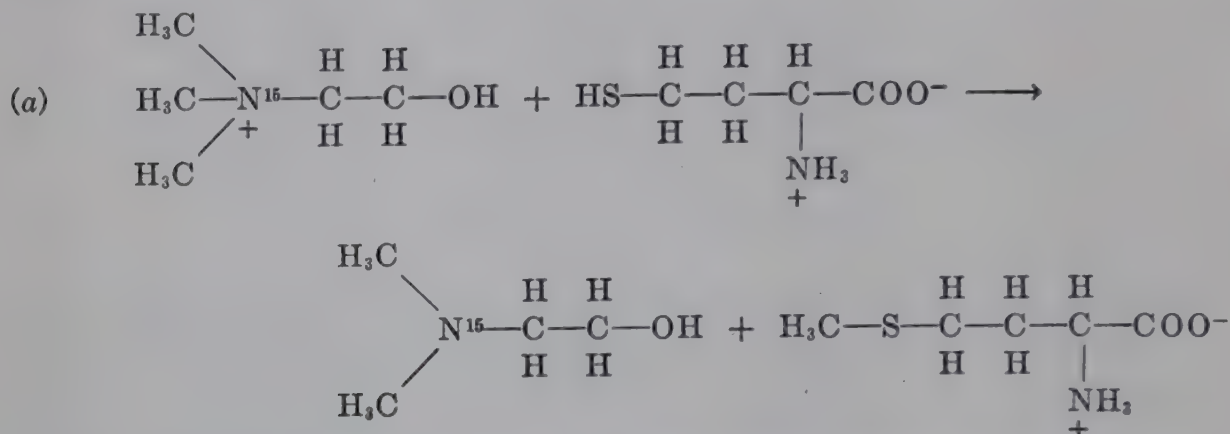
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Since the development of the concept of "labile" methyl groups in the metabolism of mammals, it has been apparent that choline is one of the naturally occurring sources of methyl groups. The evidence for this comes largely from two lines of research. One is the demonstration that when rats are fed choline labeled with deuterium in the methyl groups these labeled groups appear in methionine and in creatine which may be isolated from the body tissues (1). The other line of evidence comes from experiments with young rats in which it was shown that choline will support growth on a methionine-free diet properly supplemented with homocysteine (2). Formation of methionine from choline (or betaine) and homocysteine has been stated to be one type of transmethylation (3). There remained the question of whether the choline was a direct donor of methyl groups or whether it had to be converted to another compound before methyl transfer could occur.

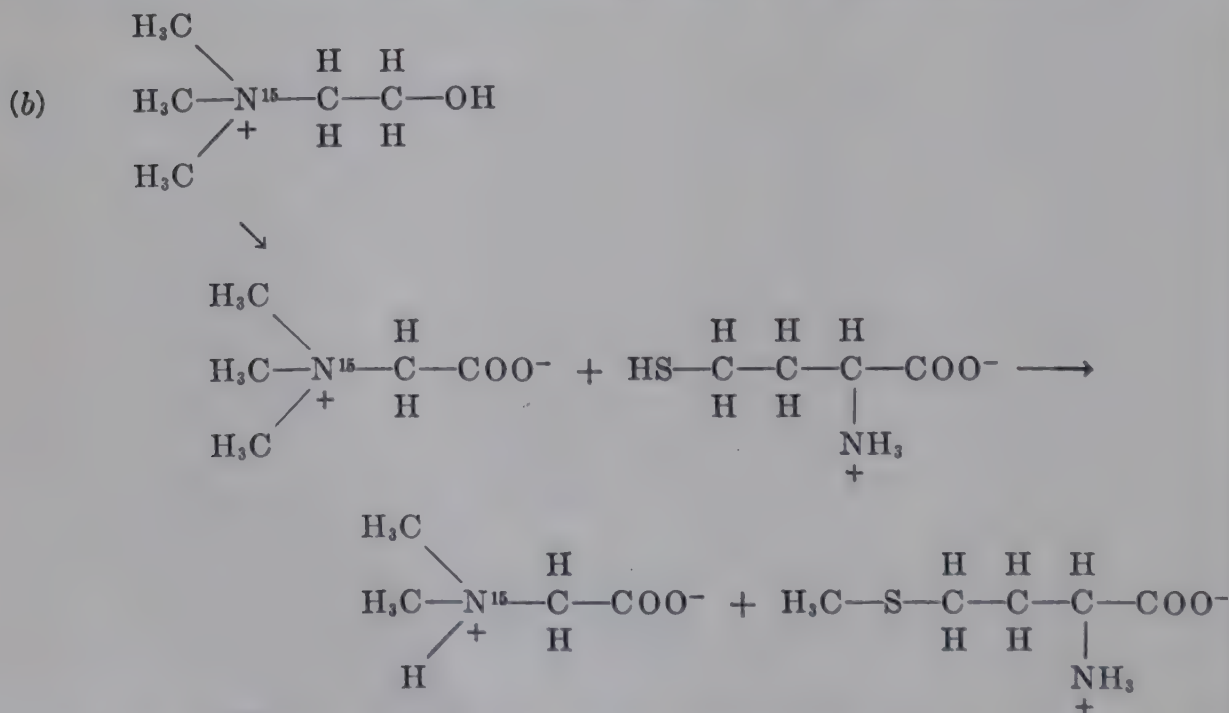
Further study of the transmethylation process, with liver slices and extracts, at first seemed to support the idea that choline was a direct methyl donor, since the formation of methionine from choline and homocysteine could occur anaerobically in such isolated systems (3). However, in these experiments betaine was more effective than choline for the formation of methionine. The possibility that choline was converted first to betaine prior to reaction with homocysteine could not be ruled out.

More recently it has been reported that liver homogenates prepared from rabbits, guinea pigs, and chicks, which do not oxidize choline, are unable to catalyze the formation of methionine from homocysteine and choline (4). Other types of experiments which throw doubt upon the ability of choline to transfer directly its methyl groups have been done with some sulfur-containing compounds, the thietins. It has been shown that dimethylthetin and propiothetin, which are analogous to betaine and alanine betaine respectively, will cause methionine to be formed when they are incubated with homocysteine in the presence of rat liver homogenates (5). They will also support the growth of young rats that are

kept on a diet lacking in methionine and choline but supplemented with homocysteine (6). On the other hand, the sulfur compound analogous to choline (sulfocholine) will not support the growth of young rats kept on a similar diet (7).



With the use of the recently described system that synthesizes methionine *in vitro* (5), it was possible to test whether choline could transfer methyl directly to homocysteine. Choline chloride was prepared which contained 30 atom per cent excess N^{15} . It was allowed to react with homocysteine in the presence of rat liver homogenate. Since it has been shown that only one of the methyl groups of choline is labile (8), direct participation of choline in the methylation of homocysteine should yield dimethylaminoethanol which contained the N^{15} of the choline (a).



On the other hand, if choline is first oxidized to betaine before methyl transfer can occur, and if betaine is the direct donor, dimethylamino-

ethanol should not be formed, but instead dimethylglycine containing N^{15} should appear as a product of the reaction. Betaine has been shown to cause methionine formation when it is incubated together with homocysteine and rat liver slices or homogenates (3). This is shown schematically (b).

In both of these formulations, which are written for illustrative purpose and not to indicate exact mechanisms, the reaction is written with homocysteine as the methyl acceptor. In the relatively crude system used, there may be other acceptors as well. Furthermore, there may be still other intermediates between choline and betaine on the one hand and the methyl acceptor on the other.

The experiments reported here show that the N^{15} of choline appeared in a fraction that had the characteristics of dimethylglycine, and negligible amounts appeared in dimethylaminoethanol.

EXPERIMENTAL

Isotopic Choline—500 mg. of NH_4Cl containing 31 atom per cent N^{15} were made to react with 1.33 gm. of paraformaldehyde (9). The resulting trimethylamine was distilled directly into ethylene chlorohydrin held at -30° . When all the amine had been transferred, the glass bomb tube containing the ethylene chlorohydrin was sealed and heated for 3 hours in a boiling water bath. The contents of the tube were washed out with absolute ethanol, and the choline chloride was precipitated by adding 10 volumes of absolute ether. It was purified by repeated solution in absolute ethanol and reprecipitation with ether. A small sample (70 mg.) was prepared for analysis in the mass spectrometer (10) and was found to contain 30.12 atom per cent excess N^{15} .

Synthesis of Methionine in Liver Homogenates—Homogenates were made from the livers of rats fasted 24 hours by homogenizing 1 part of liver with 4 parts of phosphate buffer in an all-glass homogenizer. The buffer had the following composition: 0.0128 M sodium phosphate, pH 7.4; 0.123 M NaCl; 0.005 M KCl; 0.0033 M $MgSO_4$; and 0.02 M $NaHCO_3$. Five large Warburg type vessels were set up as follows: 3.75 ml. of homogenate, 11.25 ml. of buffer, 2.5 ml. of buffer containing 3.5 mg. of homocysteine hydrochloride, and 2.5 ml. of solution containing 5.6 mg. of choline chloride in a side arm. The vessels were gassed throughout the 4 hour incubation period with N_2 or 95 per cent N_2 -5 per cent CO_2 or combinations of these gases. They were incubated at 37° with continuous gentle shaking. The solution of choline chloride was tipped into the main compartment after an initial 4 minute gassing period. After incubation, the contents of the flasks were pooled, and each flask was washed out with 5 ml. of H_2O which was added to the total volume. Trichloroacetic acid (12.5

ml. of a 45 per cent solution) was then added to deproteinize the mixture. Methionine determinations were made on 2 ml. samples of filtrate by a sensitive modification of the McCarthy-Sullivan method (5). To the remaining filtrate, 125 mg. of sodium dimethylglycine (DMG) and 0.2 ml. of dimethylaminoethanol (DAE) were added as carriers for the small amounts of these substances which might have been formed during the incubation period. In some experiments neutralized DAE was added with the choline prior to incubation; thus any small amount of DAE formation from choline would immediately mix with the large pool of added DAE, and the formed DAE would be present in the residual DAE even though there was a secondary conversion of part of it to other products.

Separation of DAE from DMG in Filtrate—The trichloroacetic acid filtrate was concentrated to about 10 ml. *in vacuo* and the concentrate was made 0.2 N with respect to HCl. It was then extracted eight times with 10 ml. portions of ether to remove trichloroacetic acid. The extracted solution was adjusted to pH 9 and then was made 0.1 N with respect to NaOH. This alkaline solution was extracted with ether in a continuous extractor for 6 hours. The ether phase contained 2.5 ml. of 1 N HCl to convert the amine into its hydrochloride. The extracted alkaline solution was saved for DMG isolation.

Isolation of DAE from Ether Phase—Ether was evaporated from the solution on a steam bath, and the amine hydrochloride was concentrated to approximately 0.5 ml. It was then treated with a small amount of anhydrous CaO; thus the resulting mixture appeared dry and crumbly. This mixture was then extracted continuously for 4 hours with ether into 200 ml. of ether¹ containing 0.55 gm. of picrolonic acid (11). Dimethylaminoethanol picrolonate began to separate out immediately, and nearly quantitative yields were obtained. The precipitate was collected on a filter and was recrystallized five or six times from absolute ethanol. Its melting point was found to be 196.5–197.3° (corrected). The melting point has been shown to be about 197° (12).

Separation and Decomposition of DMG Fraction—While it was possible to separate DAE from the mixture as the picrolonate, which is a well characterized derivative, no success was achieved in preparing a good derivative of DMG. Consequently, the following rather long purification procedure was devised. The extracted alkaline solution was centrifuged, and the precipitate was discarded. The supernatant was adjusted to pH 6.5 with 5 N HCl and the mixture was dried *in vacuo*. The resulting dry salt was extracted five times with 25 ml. portions of boiling absolute

¹ Squibb's U. S. P. ether for anesthesia was used to dissolve the picrolonic acid; absolute ether proved to be unsatisfactory for this purpose.

alcohol, and the combined extracts were filtered and again dried *in vacuo*. The residue, dissolved in 10 ml. water, was adjusted to pH 7 and was then passed through a 6×100 mm. column of Amberlite IRC-50 resin, sodium form.² The effluent was treated with 5 ml. of 30 per cent NaNO_2 and 3 ml. of glacial acetic acid at room temperature for 10 minutes, and the mixture was then heated to 90° for 30 minutes with continuous aeration to decompose the nitrous acid and to remove part of the acetic acid. Following this procedure, the flask was cooled, and the mixture was re-adjusted to pH 7 with 10 N NaOH . 3 ml. of a 20 per cent $\text{Mg}(\text{OH})_2$ suspension were added and the mixture was aerated again for 50 minutes. The entire mixture was treated with 6.6 mm of Ag_2O in the manner described by Herbst and Clarke (13). Dimethylamine derived from dimethylglycine was caught in 5 ml. of ice-cold 0.2 N H_2SO_4 . It was necessary to remove from the DMG fraction as many as possible of the nitrogenous products which might contain N¹⁵. These would include betaine and betaine aldehyde (14), as well as unchanged choline, since it has been shown that rat liver preparations catalyze the oxidation of choline to these products. Choline and betaine aldehyde are removed quantitatively by the slightly alkaline IRC-50 column. It is necessary to work with an alkaline column since HCl is liberated when choline chloride is adsorbed on the resin as furnished by supply houses. If this were to occur, dimethylglycine would also be retained on the column. From the alkaline column, DMG and betaine pass quantitatively into the effluent. Attempts to use phosphotungstic acid and Reinecke salt for separating these two substances were unsuccessful, despite the fact that betaine is readily precipitated with Reinecke salt, while DMG is not. Apparently a coprecipitation takes place.

However, DMG and betaine behave quite differently when they are treated with a silver oxide suspension at 100° .³ When DMG is boiled gently in a mixture of Ag_2O and $\text{Mg}(\text{OH})_2$, a nearly quantitative yield of dimethylamine is obtained, and this may be removed by aeration of the mixture with either hydrogen or nitrogen. On the other hand, betaine is completely stable under these conditions (13). Under the same circumstances, primary amino acids react to liberate NH_3 ; consequently, nitrous acid was used to destroy these amino acids. It was found that when the nitrous acid-treated mixture was neutralized and $\text{Mg}(\text{OH})_2$ was added some NH_3 was liberated. Therefore, the mixture was aerated for 50 min-

² A suspension of Amberlite IRC-50, as furnished by the Fisher Scientific Company, was treated with 5 N NaOH while stirring continuously. When the pH of the mixture remained constant at 8.0, the mixture was filtered, and the resin was washed with distilled water and stored damp.

³ The author thanks Dr. David B. Sprinson for suggesting this method of separating betaine from dimethylglycine.

utes prior to Ag_2O treatment. This final aeration was not carried out in Experiments 1 to 3 of Table I.

TABLE I

*N¹⁵ Content of Various Nitrogenous Fractions Isolated from Reaction Mixture**

Experiment No.	Amount of carrier added†		Total methionine synthesized	Atom per cent excess N ¹⁵ in		
	DAE	DMG		Dimethyl-aminoethanol	Fraction containing dimethylglycine	Volatile nitrogenous fraction
	ml.	mg.	γ			
1	0.2	100	146	0.009	0.094	
2	0.2	100	321	0.05	0.288	
3	0.2	125	962	0.008	0.269	
4	0.2	125	642	0.004	0.336	
5	0.2	100	560	0.009	0.32	
6	0.1	125	706	0.032	0.417	
7	0.1	125	1025	0.00	0.224	0.159
8	0.1	125	800	0.00	0.202	0.167

In *Experiment 1* the homogenate-buffer mixture was gassed for 5 minutes with 95 per cent N_2 -5 per cent CO_2 , then incubated for 3.5 hours at 38° . Carriers DAE and DMG were added after the incubation period. In *Experiment 2* the homogenate-buffer mixture was gassed continuously with 95 per cent N_2 -5 per cent CO_2 throughout a 4 hour incubation period. Carriers DAE and DMG were added after the incubation period. For *Experiment 3* the homogenate-buffer mixture was gassed with N_2 for 5 minutes, and the side arm tap was then left open to the air for 1 hour. The vessels were gassed with N_2 for 2 hours and finally with pure CO_2 for 1 hour. Carriers DAE and DMG were added after the incubation period. For *Experiment 4* the same gassing procedure was employed as in Experiment 3. Carrier DAE was added at the start of the experiment; carrier DMG was added after the incubation period. In *Experiment 5* the homogenate-buffer mixture was gassed with N_2 for 5 minutes; the taps were closed and the mixture was incubated for 3.5 hours. Carriers DAE and DMG were added after the incubation period. For *Experiment 6* the homogenate-buffer mixture was gassed with N_2 continuously for 1 hour, then with 95 per cent N_2 -5 per cent CO_2 for 3 hours. Carrier DAE was added at the start; DMG was added after the incubation period. In *Experiment 7* the homogenate-buffer mixture was gassed with N_2 continuously for 1 hour, then with 95 per cent N_2 -5 per cent CO_2 for 3 hours. Carrier DAE was added at the start; DMG was added after the incubation period. The same conditions were employed in *Experiment 8* as in Experiment 7.

* 100 ml. of homogenate-buffer containing 17.5 mg. of homocysteine hydrochloride and 28 mg. of choline chloride.

† DAE, Eastman Kodak β -dimethylaminoethyl alcohol. DMG, sodium dimethyl-glycine.

Preparation of Nitrogen Samples for Mass Spectrometer—The DAE picrolonate was decomposed with 7 ml. of $\text{N H}_2\text{SO}_4$ in a boiling water bath and, after cooling, was extracted by three shakings with 2 volumes of

ethyl acetate to remove picrolonic acid (11). The clear, colorless amine solution was then digested with an H_2SO_4 - CuSO_4 -selenium Kjeldahl digestion mixture for 16 hours. Dimethylamine sulfate solution derived from the dimethylglycine fraction was dried in an oven at 100° and likewise was digested for 16 hours. The Kjeldahl digests were steam-distilled into 0.5 ml. of 4 N H_2SO_4 ; the nitrogen was liberated with hypobromite reagent and was transferred to mass spectrometer sampling tubes (10).

Results

As one of the controls on the methods employed in this study, a filtrate was prepared from an incubated homogenate mixture to which no choline had been added. No DAE or DMG was added, and the entire procedure was carried out to see whether any volatile basic substances were formed during the final Ag_2O oxidation step. A small amount of such substances was formed, equivalent to 0.487 mg. of N in one experiment. This nitrogen would dilute the isotopic nitrogen derived from DMG in our experiments. It was confirmed that betaine did not react with Ag_2O either in pure solution or in the presence of extract prepared from liver filtrate, nor did it break down in any of the other chemical manipulations.

As shown in Table I, DAE isolated from the liver homogenate filtrate usually contained no significant amount of N^{15} . This was so regardless of whether carrier DAE was added at the beginning or at the end of the incubation period. Carrier DAE was added at the start of the experiment in order to have isotopic DAE, if it were formed from choline, mix immediately with the large non-isotopic pool. In this way even if part of the DAE were oxidized to DMG, the residual DAE isolated at the end of the experiment should contain some isotope. Actually the DAE added was nearly quantitatively recovered in all cases. In Experiments 2 and 6, a small amount of N^{15} was detected. The amount of N^{15} found was small by comparison with the amount of isotope found in the DMG fraction in the same experiment. To check the possibility that isotopic choline might break down to yield a small amount of amine in the course of the chemical manipulations, the following experiment was carried out. A liver homogenate was incubated with buffer in the usual way. Immediately after adding trichloroacetic acid, 25 mg. of isotopic choline chloride were added, together with 0.15 ml. of DAE and 125 mg. of DMG. These substances were then isolated from the filtrate in the manner described previously. Neither the DAE nor the DMG fraction contained a significant amount of N^{15} (Experiment 12, Table II). This shows that choline is stable under the conditions of the ether extraction and is removed quantitatively by IRC-50 resin.

Such small amounts of isotope as appeared in the DAE in some experi-

ments could be due to NH_3 or amines which might be formed as breakdown products of DMG. There are enzymes present in liver which convert DMG to sarcosine and this in turn may be converted to glycine (15). Sarcosine also may be enzymatically degraded to methylamine by glycine oxidase, and the same enzyme converts glycine to NH_3 and glyoxylic acid (16). Although these are aerobic processes, other hydrogen acceptors will promote these oxidations. Methylamine and NH_3 would form picrolonates which might be difficult to separate from the picrolonate of DAE

TABLE II

Control Experiments on Stability of Choline Containing N^{15} , with and without Incubation

Experiment No.	Amount of carrier added		Atom per cent excess N^{15} in		
	DAE	DMG	DAE	DMG	Volatile nitrogenous fraction
	ml.	mg.			
10	0.1	125	0.00	0.03	0.066
11	0.1	125	0.00	0.044	0.150
12	0.15	125	0.00	0.008	
13	0.2	11*		0.016	

In *Experiment 10* the homogenate-buffer-choline mixture was incubated without homocysteine, gassed with N_2 for 1 hour, then with 95 per cent N_2 -5 per cent CO_2 for 3 hours. Carrier DAE was added at the start of the experiment, while DMG was added to the filtrate. The conditions of *Experiment 11* are the same as in *Experiment 10*. In *Experiment 12* the homogenate-buffer mixture was incubated for 4 hours without homocysteine or choline. N^{15} -Choline chloride, DMG, and DAE were added to the filtrate, and the fractions were isolated in the usual manner. For *Experiment 13* the same general procedure was used as in *Experiment 12*, except that N^{15} -choline chloride was added after the DAE had been extracted and no carrier DMG was added.

* Sodium dimethylglycine added to provide enough nitrogen for mass spectrometer analysis.

by recrystallization. If CH_3NH_3 and NH_3 were formed, it should be possible to remove them from the alkaline solution prior to ether extraction. In some of the later experiments this was done by aerating the alkaline solution vigorously for 20 minutes. The volatile substances were caught in 0.1 N H_2SO_4 , converted to $(\text{NH}_4)_2\text{SO}_4$ by Kjeldahl digestion, and then analyzed for N^{15} . As is shown in Experiments 7 and 8, Table I, this fraction contained a significant amount of isotope, and the DAE which was removed subsequently contained no isotope.

In contrast to the negligible amounts of isotope usually found in the DAE, the DMG fraction always contained large amounts of N^{15} . The

quantity found was usually greater than the amount that would be expected from the methionine synthesized. Thus, in Experiment 2, 0.081 atom per cent excess N^{15} should have appeared in the DMG fraction if DMG equivalent to 321 γ of methionine had been formed. The extra amount of N^{15} which was found might be accounted for in at least two ways: (a) the methionine found by analysis does not represent all the methionine that was formed; (b) betaine contributed a methyl group to acceptors other than homocysteine, thus yielding an augmented amount of DMG.

Some evidence for a slight destruction of methionine was obtained, but the analytical method for methionine determination is not sufficiently sensitive or precise to make this certain. Liver slices will metabolize methionine by deaminizing it to the corresponding keto acid (17). Indirect evidence that betaine can yield some DMG in the absence of added homocysteine was obtained by carrying out a 4 hour experiment with the usual amount of N^{15} -labeled choline but with no homocysteine present. Under these conditions a small amount of isotope appeared in the DMG fraction (Experiments 10 and 11, Table II). A similar experiment, in which the choline was added *after* the addition of trichloroacetic acid to the homogenate, showed that choline N^{15} did not appear in the DMG fraction (Experiment 12, Table II).

All of the results make it quite certain that the formation of the DMG is in the main related to a reaction with homocysteine, and that the DMG is not a product of a reaction unrelated to methylation of homocysteine.

DISCUSSION

In none of these experiments was there an appreciable amount of N^{15} -labeled DAE formed when N^{15} -labeled choline chloride and homocysteine were incubated together with rat liver homogenate. Consequently, reaction (a) did not take place in this system. On the other hand, the relatively high isotope content in the fraction derived from DMG suggests that reaction (b) represents the manner in which methyl transfer from choline takes place; however, there may still be other intermediates in the transfer of the methyl group from betaine to homocysteine. The finding that DMG is the product of the reaction is in agreement with experiments *in vivo* in which betaine was shown to yield methyl groups for the synthesis of choline and creatine (18). In this same paper it was shown that betaine is not converted as a whole to choline; consequently, the reaction $\text{choline} \rightarrow \text{betaine}$ is irreversible, a fact which had previously been demonstrated with N^{15} -labeled betaine (19).

Since most of these experiments were carried out under anaerobic conditions, it is necessary to assume that choline oxidation is facilitated by hydrogen acceptors present in the liver homogenate. An anaerobic con-

dition was maintained so as to give every opportunity for methyl transfer from choline to occur and to retard the oxidation of choline to betaine. Many small scale experiments were performed to find the conditions under which uniformly maximal amounts of methionine were formed. Early experiments were carried out in phosphate buffer at pH 7.4 and the results were erratic. When bicarbonate was incorporated into the buffer and the vessels were gassed initially with N_2 , thus giving pH 7.8, it was found that methionine formation was greater and more uniform. Presumably the higher pH favors formation of betaine, whereas at the lower pH betaine aldehyde is produced (20). During the last 2 or 3 hours of the incubation period, the gas mixture was changed to 95 per cent N_2 -5 per cent CO_2 so as to lower the pH to 7.4. The lower pH appears to favor transfer of methyl from betaine to homocysteine.

It was also observed that better synthesis was obtained when the vessels were gassed continuously throughout the incubation period. This may be due to the removal of H_2S , for it was observed that, if the vessels were not gassed continuously, a strong odor of H_2S was always present.

There is not good agreement between the amount of methionine synthesized in these experiments and the level of N^{15} in the nitrogen derived from the DMG fraction. Possible reasons for this have already been presented. It is to be noted that in Experiments 10 and 11 the N^{15} level is higher in the volatile nitrogenous fraction than in the DMG fraction. This seeming paradox is explained by the fact that the isolated DMG nitrogen is diluted with 14 mg. of normal DMG nitrogen, added as carrier, while no carrier nitrogen was added to the volatile nitrogenous fraction.

This work was initiated with Dr. A. D. Welch of the Department of Pharmacology who helped with the synthesis of the N^{15} -choline chloride. It is a pleasure to acknowledge his continued interest and advice.

SUMMARY

Rat liver homogenates incubated with choline and homocysteine form methionine, but dimethylaminoethanol, the expected product of the demethylation of choline, is not produced. Instead dimethylglycine, the expected product of the demethylation of betaine, is formed. This suggests that in these systems choline does not lose a methyl group directly but must first be converted to betaine before methyl transfer can occur.

BIBLIOGRAPHY

1. Simmonds, S., Cohn, M., Chandler, J. P., and du Vigneaud, V., *J. Biol. Chem.*, **149**, 519 (1943).
2. du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.*, **131**, 57 (1939).

3. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **169**, 247 (1947).
4. Dubnoff, J. W., *Federation Proc.*, **8**, 195 (1949).
5. Dubnoff, J. W., and Borsook, H., *J. Biol. Chem.*, **176**, 789 (1948).
6. Maw, G. A., and du Vigneaud, V., *J. Biol. Chem.*, **176**, 1037 (1948).
7. Maw, G. A., and du Vigneaud, V., *J. Biol. Chem.*, **176**, 1029 (1948).
8. du Vigneaud, V., Chandler, J. P., Simmonds, S., Moyer, A. W., and Cohn, M., *J. Biol. Chem.*, **164**, 603 (1946).
9. Adams, R., and Marvel, C. S., *Org. Syntheses*, **1**, 531 (1941).
10. Sprinson, D. B., and Rittenberg, D., *U. S. Naval Med. Bull.*, suppl., 82 (1948).
11. Thierfelder, H., and Schulze, O., *Z. physiol. Chem.*, **96**, 296 (1916).
12. Knorr, L., and Mattes, H., *Ber. chem. Ges.*, **34**, 3482 (1901).
13. Herbst, R. M., and Clarke, H. T., *J. Biol. Chem.*, **104**, 769 (1934).
14. Mann, P. J. G., and Quastel, J. H., *Biochem. J.*, **31**, 869 (1937).
15. Handler, P., Bernheim, M. L. C., and Klein, J. R., *J. Biol. Chem.*, **138**, 211 (1941).
16. Ratner, S., Nocito, V., and Green, D. E., *J. Biol. Chem.*, **152**, 119 (1944).
17. Borek, E., and Waelsch, H., *J. Biol. Chem.*, **141**, 99 (1941).
18. du Vigneaud, V., Simmonds, S., Chandler, J. P., and Cohn, M., *J. Biol. Chem.*, **165**, 639 (1946).
19. Stetten, D., Jr., *J. Biol. Chem.*, **140**, 143 (1941).
20. Bernheim, F., and Webster, M. D., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **119**, p. xi (1937).

A REFERENCE BASE AND SYSTEM FOR ANALYSIS OF MUSCLE CONSTITUENTS*

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Analyses of tissue constituents are expressed conventionally with reference to one or more characteristics or components of the tissue itself. The following measurements comprise the usual references: wet weight, dry weight (total or fat-free), total nitrogen, phosphorus or carbon. For analysis of normal tissue any one of these references provides a useful means of estimating comparative concentrations. In analyses of abnormal tissue, however, the use of such a reference base may provide data which are grossly misleading. For example, it seems more than likely that abnormal concentrations of creatine phosphate reported to exist in muscle samples removed from patients with dystrophy are owing largely to the fact that 1 unit volume of dystrophic muscle may contain only a fraction of the mass of muscle fiber found in normal muscle; the remainder of the mass is composed largely of fat and collagen which do not contain significant amounts of creatine (1). Hoagland summarized the problem thus. "The problem of adequate bases of reference for constituents of diseased tissue in general is one of the principal barriers to the development of a rational discipline of chemical pathology. It is becoming increasingly evident that practical methods for determination of the residual mass of parenchymal cells of affected organs will have to be devised before full consideration can be given to possible alterations in concentrations of enzymes, substrates, metabolites, and metallic catalysts occurring in tissue as a result of affection by disease. Since, in the chemical analysis of diseased organs, we are going to be faced constantly with the need of knowing the net quantities of various tissue constituents, it is safe to assume that increasing attention will be paid in the future to the problem of the relation of the concentration of tissue constituents to differential cellular mass" (2).

Hoagland and his collaborators attacked the problem as it concerns muscle analysis by developing a technique for estimating the myosin content of the muscle sample as a reference base which is related directly

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to the mass of muscle fiber (3). Analyses of myosin content of muscle have been carried out in this laboratory, but the variation was great enough to make it seem probable that consistent recovery of myosin from the muscle sample had not been achieved.

To circumvent these difficulties another approach has been developed which depends upon the fact that the nitrogen content of normal muscle is owing almost entirely to its protein constituents. Of the protein found in normal muscle almost all is within the muscle cell; a small fraction, less than 2 per cent of the total wet weight, is provided by the structural substances, collagen and elastin (4). In diseased muscle, however, such supporting tissues may comprise an appreciable fraction. The problem then resolved itself into the need for a method of chemical "dissection" of the intrafibrillar protein from the protein of the supporting structures. We are indebted to Dr. Oliver H. Lowry for his welcome suggestion that the principle employed for estimation of tissue collagen and elastin (4) might be applied to this problem. This method depends upon the fact that collagen and elastin are insoluble in a weak solution of sodium hydroxide in which other cellular proteins dissolve. Thus, the solution resulting from treating muscle with alkali contains the nitrogen furnished by the muscle protein proper. In addition it contains nitrogen introduced by the nitrogen content of the interstitial fluid of the muscle and by that of the blood remaining in the vessels. A simple calculation reveals that the nitrogen contributed to the alkali solution from these sources will be less than 0.5 per cent of the total, even under the most abnormal circumstances. Furthermore, the nitrogen furnished by certain non-protein nitrogenous compounds found within the muscle cell, such as α -amino acids, creatine, nucleic acids, and phosphatides, totals some 5 per cent of all the nitrogen. From these considerations it appears that the alkali-soluble nitrogen in a sample of muscle is proportional to the amount of muscle fiber, with an approximate error of 5 per cent or less. To this portion of muscle nitrogen is assigned the term, *non-collagenous nitrogen* (NCN), and this value provides a reference base related to the mass of parenchymal cells. A similar application of this type of analysis has been reported recently for nerve (5).

The development of techniques for dispersing relatively tough tissues into homogeneous suspensions (6) and solutions provides a means for determining many muscle constituents without resort to dry or wet ashing, and the application of these techniques is presented below.

Methods

The methods to be described were developed almost exclusively with homogenates made from the triceps surae or with intact diaphragm of the rat.

A sample of whole muscle, ranging in weight from 300 to 1500 mg., was weighed rapidly in a torsion balance and homogenized in a Potter-Elvehjem apparatus in 3 to 4 ml. of distilled water and, when homogenization was complete, diluted with distilled water to a convenient known volume, usually 10 per cent (weight per volume). This diluted homogenate could be pipetted accurately in a Mohr pipette with a wide mouth and was in a form suitable for estimation of the activity of a variety of intracellular enzymes (*e.g.* succinate oxidase, cytochrome oxidase, adenosinetriphosphatase, etc.).

Non-Collagenous Nitrogen—1 volume of the 10 per cent muscle homogenate was added to 10 volumes of 0.05 N NaOH, stoppered, and the mixture allowed to stand 18 hours at room temperature. The intact dia-

TABLE I
Thirteen Consecutive Analyses of NCN of Triceps Surae of Rats

Experiment No.	Digest	Δ	Per cent variation
	γ N ₂ per ml.		
1	324	0	0
	324		
2	238	4	1.7
	242		
3	201	2	1.0
	203		
4	271	9	3.4
	269		
	262		
5	291	4	1.4
	287		
	287		
	291		

phragm of the rat was digested similarly without prior homogenization. This digest was filtered and the filtrate will be referred to below as the *alkali digest*. The completeness of digestion by weak alkali has been proved by redigestions of the washed residues; these second digests have been found to be consistently nitrogen-free. The nitrogen content of this digest was determined by the method of Koch and McMeekin (7). 1 ml. of the alkali digest was digested with H₂SO₄ in a 100 ml. Kjeldahl flask which was calibrated to contain 110 ml. Nesslerization and dilution were carried out in the same flask and the optical density measured in a Coleman junior spectrophotometer.

The reproducibility of the technique may be judged by the following experiments in which aliquots of homogenates were carried through digestion and N₂ analysis in parallel (Table I).

Analyses of NCN of thirteen consecutive triceps surae of rats gave a mean of 33.3 gm. per kilo of muscle (s.d. ± 1.09).

Phosphorus (Non-Collagenous, NCP)—Total phosphorus was determined by a modification of the Fiske-Subbarow method (8). 1 ml. of the alkali digest, usually containing 10 to 25 γ of P, was digested with 0.4 ml. of 10 N H_2SO_4 and 2 drops of fuming HNO_3 at 120–130° for 20 hours in an oven. If necessary, 2 drops of 30 per cent H_2O_2 were added and digestion continued for 2 hours more. Then 3 drops of distilled water were added and the tubes were returned to the oven for 2 hours. The usual Fiske-Subbarow technique was followed and, after development of color, density was determined at 660 m μ in the Coleman junior spectrophotometer.

Eleven determinations of phosphorus in the triceps surae of normal rats yielded a mean content of 69.7 mm (2.16 gm.) per kilo of muscle (s.d. ± 3.1). The mean of the ratio NCP:NCN was 0.065 or 1:15.4, and the standard deviation ± 0.0027 .

Determinations of muscle cations were complicated by the discovery, during recovery studies, that small amounts of glass were ground off the homogenizer, and that these fragments contributed variable and unpredictable amounts of sodium to the homogenate and introduced turbidity into the magnesium preparations. This difficulty was circumvented finally by constructing a homogenizing mortar of Lucite. With this apparatus no further contamination of the homogenate was encountered.

Potassium was determined directly from a filtrate of the alkali digest by means of a modified Berry-Chappell-Barnes internal standard flame photometer (9). This apparatus is sensitive to changes in the concentration of K of 5 $\mu\text{eq.}$ per liter, and studies of recovery of K from biological fluids yielded a mean error of 1 per cent. The alkali digest was diluted from 1:2 to 1:5 with the lithium internal standard to a final Li concentration of 200 parts per million. This dilution caused no obstruction in the spraying system of the photometer. Thirty-four analyses for K in triceps surae of normal rats of widely varying weights and ages yielded a mean concentration of 121.3 m.eq. per kilo of muscle (s.d. ± 8.7). The K content, expressed as milliequivalents per gm. of NCN, was 3.76 (s.d. ± 0.4).

Pilot experiments indicated that *sodium* could be determined in a similar fashion, except that the alkali digest was made with 0.05 N KOH which, however, contained a small but significant Na blank. Aliquots of muscle homogenates digested in parallel by NaOH and KOH have been found to contain the same amounts of NCN.

Magnesium was determined from the muscle homogenate by a slight modification of the colorimetric methods employing Titan yellow (10, 11). This method is based on the observations of Kolthoff that mag-

nesium ions in the presence of Titan yellow in an alkaline solution produce a red color. 1 gm. or less of triceps surae muscle was removed, quickly weighed, and homogenized in glass-distilled water. 5 ml. of 10 per cent homogenate were added to an equal volume of 10 per cent trichloroacetic acid, mixed, and centrifuged. The supernatant was filtered to remove small particles of precipitate which were not packed. Of this filtrate, a 2 ml. aliquot provided a sample containing 20 to 30 γ of magnesium. The total volume of the filtrate was about 7 ml., permitting triplicate determinations for each 500 mg. of muscle. The use of aliquots containing 20 to 30 γ of Mg provided a transmittance of about 60 per cent with the Coleman junior spectrophotometer at a wave-length of 540 $m\mu$. Greater concentrations of Mg may result in a flocculent precipitate on addition of Titan yellow.

To 2 ml. of filtrate in a 10 ml. volumetric flask was added 1 drop of 0.01 per cent methyl red indicator, and preliminary titration was made with 6 per cent NaOH. About 7 drops of 6 per cent NaOH usually sufficed; back-titration with weak HCl was carried out if the end-point was passed. Finer titration with 0.4 per cent NaOH was carried out to bring the indicator just to the yellow color. By means of the glass electrode, final pH has been determined to range between 11.2 and 11.4 in a series of twenty titrations. The use of 16 per cent NaOH as described by Garner (10) was noted to decrease the transmittance without increasing the spread within the desired range of 0 to 30 γ of Mg. The titrating solutions must be magnesium-free. The remainder of the procedure was as described by Kunkel *et al.* (11).

We have used duplicate blanks and duplicate standards containing 10, 20, and 30 γ of Mg, constructing a curve for each day's analysis. Color was stable for at least 1 hour. After an hour or more there appeared a flocculent color lake which could be resuspended by shaking. Resuspension after 24 or more hours produced a density equal to that found at the time of original color development.

The results obtained on analysis for Mg of both right and left triceps surae muscles of a series of ten normal rats are tabulated below in detail because of the paucity of such data in the literature (Table II). These determinations were made on duplicate aliquots of homogenate of each muscle, each aliquot being determined in triplicate.

Chloride content may be measured by an open Carius digestion of the alkali digest and Volhard titration, but it has been found that, with relatively small amounts of tissue available, the Conway microdiffusion method proved easier (12).

Aliquots of muscle homogenates have been used to assay activity of succinate oxidase, cytochrome oxidase, and adenosinetriphosphatase.

Determination of water content has been made by delivering a meas-

ured volume of 10 per cent muscle homogenate into small tared tubes. The homogenate was dried at 105° for 48 hours and the water content estimated by weight difference. Six analyses yielded an average water content of 76.8 per cent (s.d. \pm 1.5).

TABLE II
Magnesium Content of Triceps Surae Muscle in Rats
Expressed as milliequivalents per kilo of muscle (wet weight).

Rat No.	Left	Right	Δ	Average
1	24.8	24.3	0.5	24.6
2	25.9	27.6	1.7	26.8
3	23.8	25.0	1.2	24.4
4	25.4	23.8	1.6	24.6
5	23.1	22.2	0.9	22.7
6	23.6	22.4	1.2	23.0
7	22.8	22.4	0.4	22.6
8	20.5	20.1	0.4	20.3
9	24.0	24.8	0.8	24.4
10	21.9	21.5	0.4	21.7
Mean.....	23.6	23.4	0.9	23.5
S.D.....	1.6	2.2		1.8

Each value represents the average of six determinations.

TABLE III
Comparison of Values Obtained by Differing Techniques (Rat)
The values are expressed as milliequivalents per kilo.

	Digestion	Ashing
K.....	121.3	113.3*
Mg.....	23.5	22.0†
P.....	69.7	72.3‡

* Lowry, O. H., Hastings, A. B., Hull, T. Z., and Brown, A. N., *J. Biol. Chem.*, **143**, 271 (1942).
† Conway, E. J., *Biol. Rev. Cambridge Phil. Soc.*, **20**, 56 (1945).
‡ Darrow, D. C., Schwartz, R., Iannucci, J. F., and Coville, F., *J. Clin. Invest.*, **27**, 198 (1948).

DISCUSSION

The application of the homogenate technique provides an analytical method for muscle which obviates the necessity for ashing. Comparison of values obtained by alkali digestion and by ashing are presented in Table III. The employment of the differential solubility of supporting

tissues (collagen and elastin) and muscle cells in weak alkali enables a chemical "dissection" to be made which separates these two tissues.

It is altogether probable that in certain abnormal states the usual quantitative gravimetric relationships of N_2 to intracellular protein, and each of these to cell mass, may be distorted. For this reason, the selection of NCN as a reference base may not solve the original problem, but simply contributes toward a solution by exclusion of supporting tissues.

The reasonable assumption has been made throughout that the alkali digest contains a representative concentration of ions furnished by the intracellular and extracellular stores. The remote possibility that the collagen-elastin residue adsorbs selectively significant amounts of one or another ion has not been excluded.

In the development of the methods described here the contribution of lipid N_2 to the NCN has been neglected. In normal rat muscle the fat content is less than 1 per cent and the N_2 content of crude depot fat is less than 0.6 per cent.¹ Under these circumstances the error introduced by expressing concentrations with reference to NCN, without defatting, is insignificant. In atrophied or diseased muscle such an assumption is unwarranted. In this case defatting has been carried out by repeated agitation with ethyl ether and then with petroleum ether, decanting and warming the homogenate to drive off residual ether prior to alkaline digestion.

Although insufficient data have been accumulated to insure validity, preliminary observations make it seem likely that the methods described above are applicable to tissues other than muscle. It need be recalled, however, that factors which permitted neglect of intracellular non-protein N_2 and of extracellular contamination in the case of skeletal muscle might not necessarily operate in certain other tissues.

SUMMARY

1. Digestion of muscle homogenates in weak alkali provides a solution of muscle fibers freed from their supporting tissues, collagen and elastin. The N_2 content of this digest furnishes a reference base for estimation of muscle constituents in normal and diseased tissue.

2. The alkali digest is suitable for direct flame photometric estimation of either potassium or sodium content and for colorimetric estimation of phosphorus. The homogenate furnishes a preparation from which estimation of magnesium may be made colorimetrically. These methods obviate the necessity for either wet or dry ashing.

¹ Unpublished observations.

BIBLIOGRAPHY

1. Nevin, S., *J. Neurol. and Psychiat.*, **1**, 120 (1938).
2. Hoagland, C. L., in *Currents in biochemical research*, New York (1946).
3. Hoagland, C. L., *Advances in Enzymol.*, **6**, 193 (1946).
4. Lowry, O. H., Gilligan, D. R., and Katersky, E. M., *J. Biol. Chem.*, **139**, 795 (1941).
5. Abercrombie, M., and Johnson, M. L., *J. Neurol., Neurosurg., and Psychiat.*, **9**, 113 (1946).
6. Potter, V. R., *Methods in medical research*, Chicago (1948).
7. Koch, F. C., and McMeekin, T. L., *J. Am. Chem. Soc.*, **46**, 2066 (1924).
8. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
9. Berry, J. W., Chappell, D. G., and Barnes, R. B., *Ind. and Eng. Chem.*, **18**, 19 (1946).
10. Garner, R. J., *Biochem. J.*, **40**, 828 (1946).
11. Kunkel, H. O., Pearson, P. B., and Schweigert, B. S., *J. Lab. and Clin. Med.*, **32**, 1027 (1947).
12. Conway, E. J., *Micro-diffusion analysis and volumetric error*, London (1947).

THE EFFECT OF SULFATHALIDINE ON THE EXCRETION OF VITAMIN B BY RUMINANTS*

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It is an established fact that some vitamins of the vitamin B complex are synthesized by microorganisms in the intestinal tract. Mitchell and Isbell (1) have shown that such activity occurs in the rat. Similar results have been reported for the human with respect to thiamine (2-4), riboflavin (4-6), and other vitamins (4). With respect to adult ruminants it is generally assumed that the synthetic activity of microorganisms makes these animals at least partially independent of a dietary source of some of the water-soluble vitamins (7).

The administration of sulfonamides has become a valuable means of studying the requirements for certain vitamins (8). By this means the facts have been established that biotin and folic acid are essential for the rat (9-13). A decreased fecal excretion of pantothenic acid when rats were fed sulfonamides has been reported (13). With humans the administration of sulfathalidine resulted in a decreased fecal excretion of *Lactobacillus casei* factor and biotin, while there was no decrease in any of the urinary vitamin levels (14). In a study with calves it was found that the incorporation of sulfathalidine in the diet did not affect growth or the urinary excretion of nicotinic acid and its metabolites (7). The constancy of excretion of these compounds on a nicotinic acid-free ration indicated synthesis of nicotinic acid in the tissues. Fecal excretion of this vitamin, however, was not studied and it appears that data are lacking on the effect of sulfonamides upon the fecal elimination of vitamin B by ruminants. Since the synthetic activity of the rumen or intestinal microorganisms appears to be of considerable importance in ruminant nutrition, it seemed desirable to carry out a study with the sulfonamide administration technique of the vitamin B synthesis in the digestive tract of the bovine species. It is the purpose of this report to present the results of an investigation into the effects of sulfathalidine on the urinary and fecal excretion, by ruminants, of nicotinic acid, thiamine, pantothenic acid, and riboflavin.

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EXPERIMENTAL

General—Four heifers were used in this study. Cows 1, 2, and 3 were Guernseys, while Cow 4 was a Holstein. All were approximately 2 years of age. The animals were maintained in metabolism stalls, making possible the regular collection of 24 hour urine and feces samples. Aliquots of these collections were taken daily and preserved (by freezing for the feces and by refrigeration at a temperature just above freezing for the urine) until five consecutive 24 hour samples had been obtained. The five aliquots were then pooled into one sample for analysis. Three such 5 day periods constituted an experimental "series" on any particular ration. A preliminary feeding period of at least 1 week served to adjust the animals to their rations which, in all cases, consisted solely of hay and water. Analysis of the roughage for various B vitamins indicated the following average daily intakes: 252 mg. of nicotinic acid, 4.7 mg. of thiamine, 60 mg. of pantothenic acid, and 67 mg. of riboflavin.

The sulfathalidine, in pill form, was administered orally, by balling-gun, twice daily. The dosage amounted to 56 gm. per day for Cows 3 and 4 and 40 gm. per day for Cow 1. Administration of this compound was carried on for at least a week before collections of urine and feces were made. Although only three of the four animals received the sulfonamide, the "control" data obtained with the fourth animal (Cow 2) have been included in Tables I to VII, since this animal received the same ration and was treated in the same manner as the other three when they were receiving the roughage without sulfathalidine.

Analytical Methods—All vitamin analyses were made by microbiological procedures. Nicotinic acid was determined by a modification (15) of the procedure of Snell and Wright (16). Pantothenic acid and riboflavin were determined by the procedures ((15) pp. 35–41) involving the use of *L. casei*, while the *Lactobacillus fermentum* assay (17) was used for thiamine. This microbiological thiamine assay was chosen because the sensitivity and relative speed of the method make it a desirable procedure for routine analyses. Furthermore, in a preliminary investigation it was determined that this method is applicable to the quantitative determination of thiamine in urine, feces, and milk (Table I), and that consequently it is of value in excretion studies.

A previous study (18) showed that the nicotinic acid assay would not be affected by the amount of sulfathalidine that could possibly be carried over into the assay samples, and the *L. casei* procedures have been found to be even less susceptible to a sulfonamide effect. Tables II and III show that sulfonamides, if present in sufficient concentration, inhibit the growth of *L. fermentum*. In the excretion study the greatest possible concentration of sulfathalidine that could have been present in any assay samples was not sufficient to have an effect upon the thiamine determinations. The

TABLE I
Thiamine in Urine, Feces, and Milk

Sample	Cow No.	Thiamine		Recovery	24 hr. thiamine excretion
		Added per gm.	Found per gm.		
		γ	γ	per cent	mg.
Feces.....	1	0.0	0.50		6.33
	1	0.5	1.02	104	
	2	0.0	0.33		4.68
	2	0.5	0.80	94	
Urine.....	1	0.0	0.70		4.04
	1	0.5	1.15	90	
	2	0.0	0.32		3.43
	2	0.5	0.78	92	
Milk (pasteurized).....		0.0	0.57		
		0.5	1.11	108	

TABLE II
Growth of Lactobacillus fermentum in Presence of Sulfathalidine

The recorded values are turbidity readings.

Thiamine	Standard curve	Sulfathalidine per tube				
		2.0 mg.	4.0 mg.	6.0 mg.	8.0 mg.	10.0 mg.
γ						
0.01	40	40	42	40	40	25
0.02	78	82	80	80	82	55
0.03	122	122	118	120	105	70
0.04	160	160	162	140	140	100
0.05	198	200	190	158	155	100

TABLE III
Effect of p-Aminobenzoic Acid on Suppression of Growth Caused by Sulfapyridine

The recorded values are turbidity readings.

Thiamine	Standard curve	10 mg. sulfapyridine per tube		
		No p-aminobenzoic acid	Added p-aminobenzoic acid	
			10 γ	100 γ
γ				
0.01	35	10	32	34
0.02	70	20	68	68
0.03	107	38	110	105
0.04	142	62	142	138
0.05	174	95	170	172

inhibitory effects of higher sulfonamide concentrations can, if necessary, be prevented by increasing the concentration of *p*-aminobenzoic acid in the basal medium, as shown for sulfapyridine in Table III.

RESULTS AND DISCUSSION

Tables IV to VII present the average excretion values found for the four vitamins studied. Both the normal values and those obtained when sulfa-

TABLE IV
Average Nicotinic Acid Excretions

The values are given in mg. per day.

Cow No.	Without sulfathalidine			With sulfathalidine		
	Feces	Urine	Total	Feces	Urine	Total
1	33.0	18.7	51.7	29.0	15.9	44.9
2	34.2	19.1	53.3			
3	33.0	30.7	63.7	28.7	22.1	50.8
4	32.8	23.1	55.9	31.9	20.7	52.6
Average..	33.3	22.9	56.2	29.9	19.6	49.5

Average daily intake, 252 mg. of nicotinic acid.

TABLE V
Average Thiamine Excretions

The values are given in mg. per day.

Cow No.	Without sulfathalidine			With sulfathalidine		
	Feces	Urine	Total	Feces	Urine	Total
1	3.0	5.5	8.5	1.6	3.3	4.9
2	4.1	4.6	8.7			
3	4.1	4.1	8.2	2.0	3.8	5.8
4	5.4	4.3	9.7	1.3	3.0	4.3
Average..	4.2	4.6	8.8	1.6	3.4	5.0

Average daily intake, 4.7 mg. of thiamine.

thalidine was administered are recorded. It is seen that sulfathalidine caused only a slight decrease in the excretion of nicotinic acid. This effect, although consistent, was not great, and its significance is lessened when one considers that the daily intake of this vitamin amounted to 252 mg. It appears that intestinal or rumen synthesis of nicotinic acid is not of great importance to the bovine species, especially since tissue synthesis of this vitamin (7) probably occurs.

The excretion of thiamine was markedly decreased when sulfathalidine was fed. The greatest decrease occurred in the feces, where the average elimination was reduced by 62 per cent. Statistically, this decrease is highly significant. Since the excretion values, without sulfathalidine, were considerably greater than the daily intake (4.7 mg.), it is evident that synthesis of this vitamin occurs in the digestive tract. Undoubtedly, suppression of this synthesis by sulfathalidine accounts for the decreased ex-

TABLE VI
Average Pantothenic Acid Excretions

The values are given in mg. per day.

Cow No.	Without sulfathalidine			With sulfathalidine		
	Feces	Urine	Total	Feces	Urine	Total
1	10.8	82.0	92.8	9.7	70.4	80.1
2	16.8	77.9	94.7			
3	14.9	110.4	125.3	10.5	65.4	75.9
4	20.4	61.6	82.0	20.0	65.5	85.0
Average..	15.6	83.0	98.6	13.4	67.1	80.5

Average daily intake, 60 mg. of pantothenic acid.

TABLE VII
Average Riboflavin Excretions

The values are given in mg. per day.

Cow No.	Without sulfathalidine			With sulfathalidine		
	Feces	Urine	Total	Feces	Urine	Total
1	12.9	11.7	24.6	11.0	10.4	21.4
2	12.5	10.4	22.9			
3	14.1	11.2	25.3	13.3	8.4	21.7
4	12.9	11.2	24.1	12.2	10.4	22.6
Average..	13.1	11.1	24.2	12.2	9.7	21.9

Average daily intake, 67 mg. of riboflavin.

cretion which resulted when the drug was administered. It appears, therefore, that synthesis of thiamine by rumen or intestinal microorganisms is of considerable significance in bovine nutrition.

The administration of sulfathalidine had no statistically significant effect upon the excretion of pantothenic acid. However, since the total excretion was greater than the dietary intake (60 mg.), it appears that pantothenic acid is synthesized in the digestive tract. This synthesis could be of considerable importance in ruminant nutrition.

Riboflavin excretion was not significantly affected by the administration of sulfathalidine. Since daily intake of this vitamin (67 mg.) greatly exceeded total excretion, it is not possible, from these data, to assess the value of rumen synthesis of riboflavin in the nutrition of this species.

SUMMARY

Oral administration of sulfathalidine to heifers resulted in a decreased fecal excretion of thiamine. Statistically, this decrease was highly significant. The urinary excretion of thiamine and the fecal and urinary excretions of nicotinic acid, pantothenic acid, and riboflavin were not significantly affected. Total excretions of thiamine and of pantothenic acid were considerably greater than dietary intakes, indicating rumen or intestinal synthesis of these vitamins.

The microbiological assay for thiamine with *Lactobacillus fermentum* is readily applicable to the quantitative determination of thiamine in feces, urine, and milk, and consequently is of value in excretion studies.

BIBLIOGRAPHY

1. Mitchell, H. K., and Isbell, E. R., *Univ. Texas Pub.*, No. 4237, 125 (1942).
2. Najjar, V. A., and Holt, L. E., Jr., *J. Am. Med. Assn.*, **123**, 683 (1943).
3. Hathaway, M. L., and Strom, J. E., *J. Nutr.*, **32**, 1 (1946).
4. Denko, C. W., Grundy, W. E., Wheeler, N. C., Henderson, C. R., Berryman, G. H., Friedemann, T. E., and Youmans, J. B., *Arch. Biochem.*, **11**, 109 (1946).
5. Najjar, V. A., Johns, G. A., Medairy, G. C., Fleischman, G., and Holt, L. E., Jr., *J. Am. Med. Assn.*, **126**, 357 (1944).
6. Hathaway, M. L., and Lobb, D. E., *J. Nutr.*, **32**, 9 (1946).
7. Johnson, B. C., Wiese, A. C., Mitchell, H. H., and Nevens, W. B., *J. Biol. Chem.*, **167**, 729 (1947).
8. Anonymous, *Nutr. Rev.*, **2**, 221 (1944).
9. Nielsen, E., and Elvehjem, C. A., *J. Biol. Chem.*, **145**, 713 (1942).
10. Welch, A. D., and Wright, L. D., *J. Nutr.*, **25**, 555 (1943).
11. Wright, L. D., and Welch, A. D., *J. Nutr.*, **27**, 55 (1944).
12. Schweigert, B. S., Teply, L. J., Greenhut, I. T., and Elvehjem, C. A., *Am. J. Physiol.*, **144**, 74 (1945).
13. Miller, A. K., *J. Nutr.*, **29**, 143 (1945).
14. Grundy, W. E., Freed, M., Johnson, H. C., Henderson, C. R., Berryman, G. H., and Friedemann, T. E., *Arch. Biochem.*, **15**, 187 (1947).
15. Anonymous, *Research today*, Indianapolis, **3**, 37 (1946).
16. Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **139**, 675 (1941).
17. Sarett, H. P., and Cheldelin, V. H., *J. Biol. Chem.*, **155**, 153 (1944).
18. Teeri, A. E., and Josselyn, D., *J. Biol. Chem.*, **177**, 23 (1949).

REHYDRO VITAMIN A, THE COMPOUND FORMED FROM ANHYDRO VITAMIN A IN VIVO*

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Anhydro vitamin A is the unsaturated hydrocarbon formed when vitamin A loses a molecule of water (1). This occurs with extreme ease when a solution of vitamin A alcohol in anhydrous solvent is treated with a trace of catalyst, such as a strong mineral acid. The resulting compound has six conjugated double bonds in place of the original five which, apparently, have shifted from their normal position. The formation of anhydro vitamin A, as postulated by Meunier *et al.* (2), involves an ionization of vitamin A in the presence of acid, followed by a shift of the positive charge to a carbon atom in the ionone ring where the molecule eventually regains its electrical neutrality by losing a proton from this carbon atom according to the accompanying scheme.

The early work of Embree (3) on crude concentrates of anhydro vitamin A indicated that this material had no detectable growth-promoting power. However, a bioassay on the first crystalline preparation (1) showed a biological potency of about 15,000 U. S. P. units per gm., approximately 0.4 per cent of the activity of crystalline vitamin A alcohol. It was thought that this slight activity might be caused by contamination with a small amount of unchanged vitamin A; therefore two more crystalline preparations were made. These were scrupulously purified by repeated chromatographic adsorption and recrystallization. Both were found to have the same degree of biological activity as the first preparation, and this activity could, therefore, be considered an inherent property of the compound.

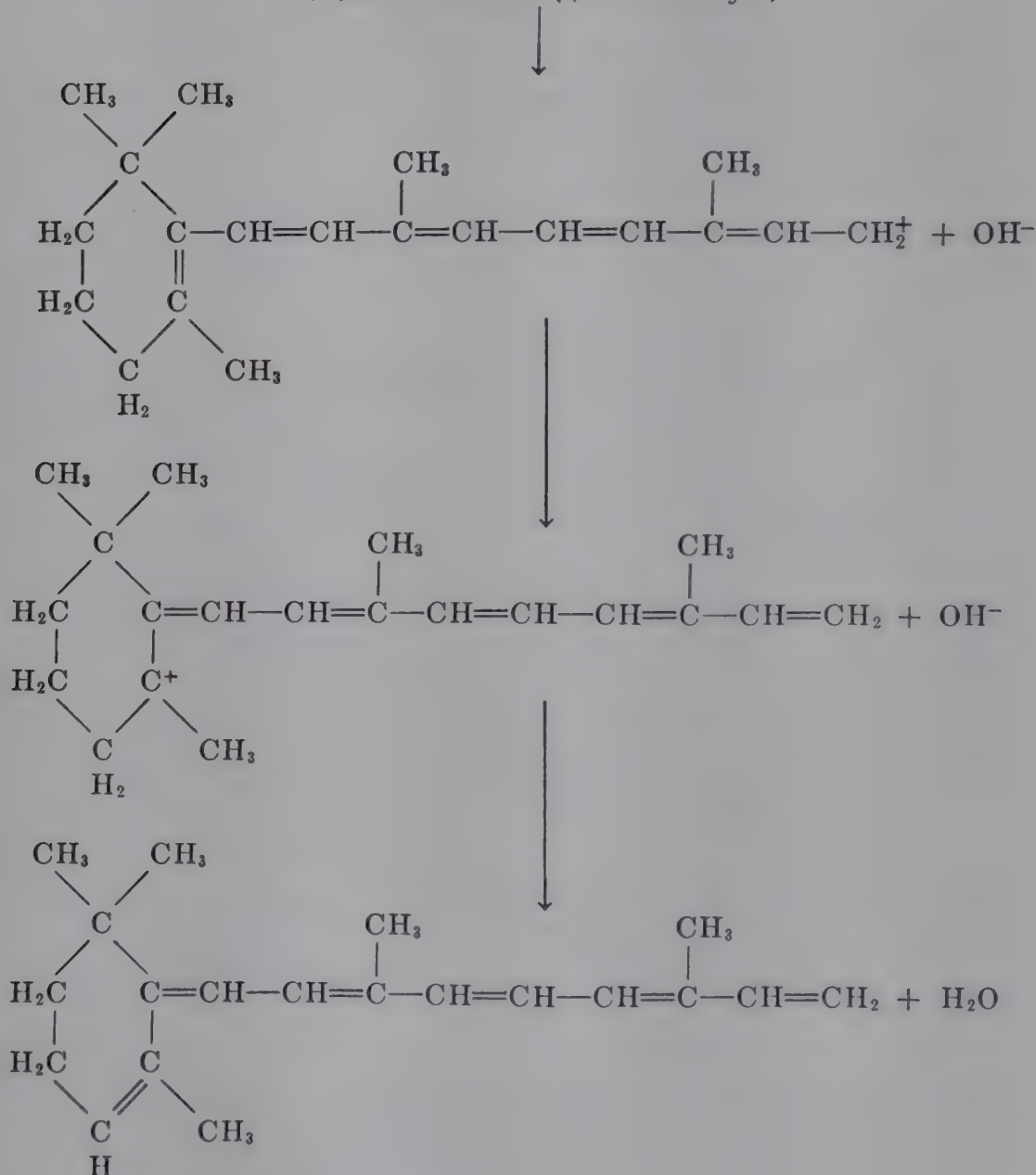
The questions with which the present paper is concerned then arose. Does anhydro vitamin A *per se* exert physiological activity, or is a small amount of it reconstituted into vitamin A by the animal body, or is it changed into an entirely new compound possessing some degree of vitamin A activity? The experimental work described below shows that a new compound is formed *in vivo* which is intermediate in activity between vitamin A and the ingested anhydro vitamin A.

The general experimental procedure was as follows: Vitamin A-de-

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ficient rats were fed crystalline anhydro vitamin A at a level high enough to produce some liver storage of the anhydro vitamin A derivative. At the end of approximately 3 weeks the animals were sacrificed and the livers removed and extracted with ethyl ether. No anhydro vitamin A or vitamin A could be detected in the liver extract. However, a com-

Vitamin A alcohol (+ acid catalyst)



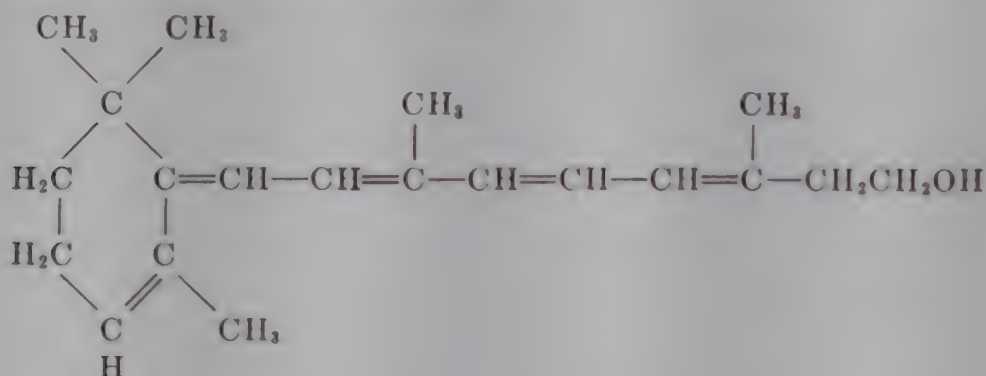
Anhydro vitamin A

pound possessing certain characteristics of both these substances was found. When this material was fed to vitamin A-deficient rats, it was found to have about 20 times the activity of the original anhydro vitamin A.

The new compound produces a blue color with antimony trichloride with an absorption maximum at about 612 mμ, in the same region as the colors

given by anhydro vitamin A, vitamin A alcohol, and other derivatives of vitamin A such as its esters and ethers. Since these compounds all have the same blue color extinction on a molecular basis, it seems reasonable to assume that the new compound behaves in the same manner, thus affording a basis for the quantitative biological comparison of this substance with crystalline vitamin A and anhydro vitamin A.

Since only a tiny amount of this compound greatly diluted with extraneous fatty material was obtained, rather indirect observations were made in an attempt to elucidate its structure. The results of these studies indicate that the new substance may be a structural isomer of vitamin A, having the formula shown in the diagram.



Rehydro vitamin A

Because of the manner in which this compound is apparently formed, the name "rehydro vitamin A" is proposed. It is conjectured that the vitamin A-deficient animal, in its attempt to utilize anhydro vitamin A, is able to add the elements of water to a small portion of the anhydro vitamin A, but is incapable of shifting the double bonds to their normal position. Thus, the animal effects a partial return toward the normal vitamin A structure and in so doing effects only a partial return toward the high activity of vitamin A.

EXPERIMENTAL

Preparation of Anhydro Vitamin A—The method of preparing anhydro vitamin A originally used by Edisbury *et al.* (4) consists of dehydrating vitamin A alcohol in N/30 anhydrous alcoholic hydrogen chloride solution. We have shown (1) that the concentration of vitamin A must be kept below 1 per cent to obtain a good yield. The preparation of a sizable quantity of anhydro vitamin A by this method involves a cumbersome volume of acidic alcohol solution. A new method suitable for the preparation of large amounts of anhydro vitamin A was devised as follows: 100 gm. of a saponified vitamin A concentrate with $E_{1\text{cm}}^{1\%}$ (328 m μ) = 1340 were dissolved in 600 ml. of benzene in a 1 liter round bottomed flask fitted with a condenser and water-collecting side arm. The solution was re-

fluxed for 15 minutes but no water could be seen in the side arm. 3 mg. of *p*-toluenesulfonic acid were added, and droplets of water immediately began to collect in the measuring tube. The theoretical amount of vitamin A present was approximately 75 gm. or 0.26 mole, which should yield 4.7 ml. of water. At the end of 2 hours, 4.6 ml. had been collected and the evolution of water had apparently ceased.

The benzene solution was cooled to room temperature and poured onto a column containing 900 gm. of aluminum oxide (Merck, according to Brockmann). The anhydro vitamin A was the most weakly adsorbed material and formed a bright yellow band which was washed through the column with Skellysolve F. The solvent was removed under nitrogen, leaving 62.5 gm. of orange oil, exhibiting absorption maxima in the ultraviolet at 351, 368, and 390 $m\mu$ with $E_{1\text{ cm.}}^{1\%}$ (368 $m\mu$) = 1800. This material was chromatographed again on a column of magnesia (Westvaco) containing 30 per cent Celite. The anhydro vitamin A fraction consisted of 44.0 gm. of viscous orange oil with $E_{1\text{ cm.}}^{1\%}$ (369 $m\mu$) = 2290. This material was dissolved in 175 ml. of Skellysolve F and after storage for 3 days at -30° yielded 11.2 gm. of crystals. Two recrystallizations from 30 per cent solutions in Skellysolve F gave a final yield of 5.5 gm. of orange prisms which melted at $76.5-77.5^\circ$. The ultraviolet absorption curve showed three maxima at 351, 371, and 392 $m\mu$ with $E_{1\text{ cm.}}^{1\%}$ = 2540, 3680, and 3200. The crystals were sealed in ampuls under a vacuum and stored at -30° for future use.

Toxicity of Anhydro Vitamin A—The amount of rehydro vitamin A stored in the liver was very small compared to the amount of anhydro vitamin A fed, and it was therefore desirable to feed as large a quantity as possible without producing toxic symptoms.

5 mg. of crystalline anhydro vitamin A were fed daily in olive oil solution to each of eighteen male rats, 6 weeks old, which had received a vitamin A-free diet for a period of 3 weeks since weaning. At this level toxic effects were noted which did not appear on a daily dose of 2.5 mg.

One rat died after 9 days on the experiment. The bladder was distended with urine and both testes showed hemorrhage. Of the three animals which died on the 10th day, two showed hemorrhage in the epididymides. One rat died on the 12th day, showing a distended bladder and hemorrhage in one testis and both epididymides. The thirteen remaining rats were killed on the 13th day. In twelve of the animals there were hemorrhages in either the testes or epididymides. In one there was subcutaneous hemorrhage in the abdominal region, while in another a subcutaneous hemorrhage occurred near the right shoulder.

In a parallel experiment with a vitamin A concentrate, approximately 5.6 mg. of vitamin A were fed daily to each of six weanling rats for 5

weeks. No deaths occurred until the 33rd day of the experiment. By the 10th day, two of the rats were beginning to lose hair around the mouth, and, by the 21st day, four of the animals had spontaneously broken one or more legs. Similar toxic effects of high potency fish liver oils were observed by Vedder and Rosenberg (5). It appears that anhydro vitamin A has a toxic effect somewhat different from that of vitamin A.

Feeding of Anhydro Vitamin A—Vitamin A-deficient rats grew well when a daily supplement of approximately 0.1 mg. of anhydro vitamin A was added to the diet, but no detectable amount of material was stored in the liver at this level. In order to feed anhydro vitamin A at a level at which some liver storage would be obtained, but no symptoms of toxicity would appear, a daily dose of 1.0 mg. was used.

In the first feeding experiment, 55 male weanling rats were placed on a vitamin A-free and low vitamin E diet for a period of 14 days, at which time the absence of any stored vitamin A was assumed, since the liver extracts from five animals gave no blue color with antimony trichloride. The rats were then given a daily supplement of 1.0 mg. of crystalline anhydro vitamin A and 0.5 mg. of mixed tocopherols for a period of 18 days. (A preliminary experiment had shown that the simultaneous supplement of tocopherols increased the storage of vitamin A-active material in the liver by about 25 per cent.) During this 18 day period the animals gained an average of 60 gm. in weight. They were killed on the 19th day and the livers were removed, weighed, and stored at -30° until they were analyzed.

Examination of Livers for Vitamin A-Active Material

Extraction—The livers from the above animals (50 livers, 384 gm.) were minced in a Waring blender with 200 ml. of 0.05 N aqueous KOH and 50 ml. of ethanol. The suspension was extracted four times with 250 ml. portions of ethyl ether. The combined ether solutions were washed, dried over sodium sulfate, and filtered. Removal of the solvent under nitrogen left 11.0 gm. of a light yellow oil.

Saponification—A 7.0 gm. portion of this oil was saponified by warming under nitrogen to 80° for $\frac{1}{2}$ hour in a solution of ethyl alcohol containing 2.0 gm. of KOH. The solution was diluted with water and extracted three times with ethyl ether. The combined ether extracts were washed, dried, and the solvent removed under nitrogen. The residue consisted of 0.435 gm. of viscous red oil.

Ultraviolet Absorption Measurement—Ultraviolet absorption curves were determined for both the whole oil from rat liver and for the unsaponifiable portion. The whole oil showed very strong extraneous absorption below 300 $m\mu$, most of which disappeared upon saponification. Otherwise, the

two curves were similar in shape, with absorption maxima at 351 and 369 $m\mu$, and an inflection at about 330 $m\mu$. $E_{1\text{ cm.}}^{1\%}$ (351 $m\mu$) for the whole oil was 0.59 and for the unsaponifiable portion 10.2 (Fig. 1). The absorption curves of crystalline vitamin A alcohol and crystalline anhydro vitamin A are also reproduced in Fig. 1 for comparison. The ultraviolet curve of this new product is very similar in appearance to "isoanhydro vitamin A" (1) which is formed by prolonged treatment of vitamin A in

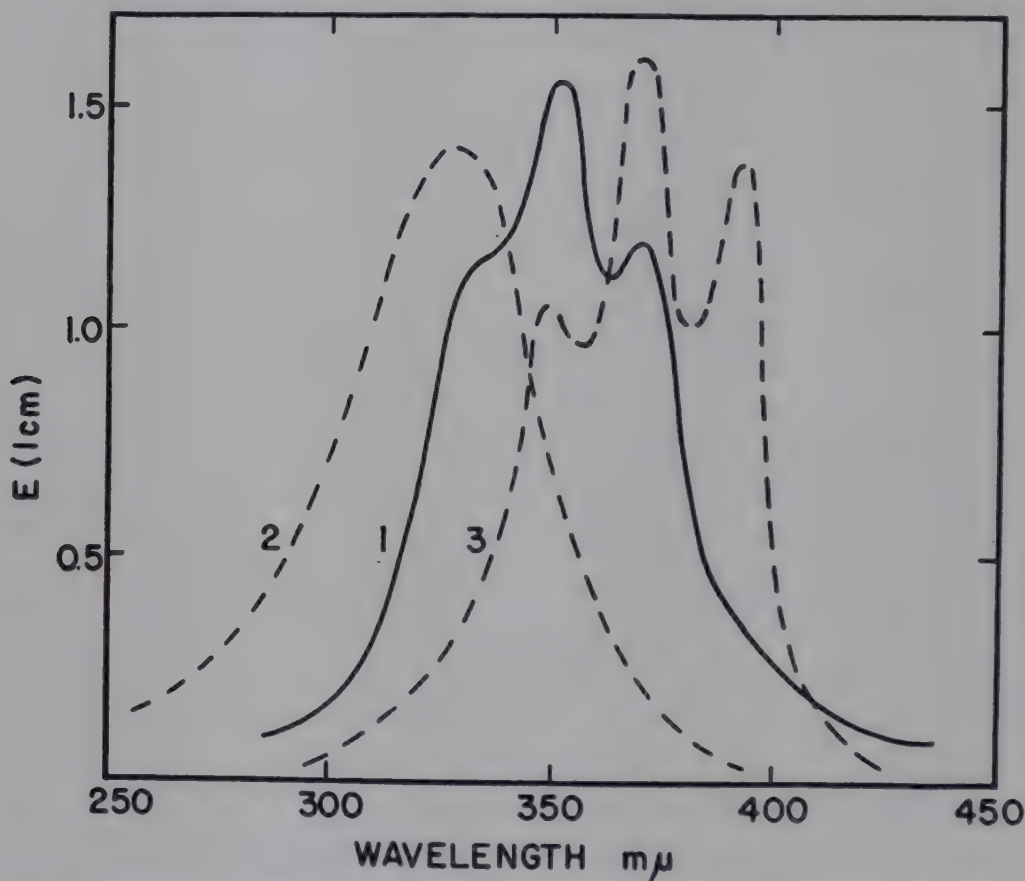


Fig. 1. Ultraviolet absorption spectra in ethanol. Curve 1, 0.152 per cent solution of saponified liver oil extract from rats fed anhydro vitamin A; Curve 2, 0.0008 per cent solution of crystalline vitamin A alcohol; Curve 3, 0.00044 per cent solution of crystalline anhydro vitamin A.

dry alcoholic hydrogen chloride. However, its other physical properties described below are totally different, which precludes the possibility that the two substances are identical.

Antimony Trichloride Reaction Product—The rat liver oil was found to give a blue color with antimony trichloride solution, and the absorption curve of this reaction product was determined on a General Electric recording visual spectrophotometer. The absorption spectra of the various fractions all showed only a single band with a sharp maximum at 612 $m\mu$. $E_{1\text{ cm.}}^{1\%}$ (612 $m\mu$) for a fraction of chromatographically purified whole rat liver oil was 3.33. In the ultraviolet region, this fraction had

a value of 2.30 for $E_{1\text{ cm}}^{1\%}$ (351 $m\mu$). Thus, the ratio $E(612\text{ }m\mu)$ to $E(351\text{ }m\mu)$ for this new substance is 1.45, about the same as the ratio $E(620\text{ }m\mu)$ to $E(372\text{ }m\mu)$ for anhydro vitamin A.

Chromatographic Adsorption—1 gm. of the whole rat liver oil was adsorbed on a column containing 20 gm. of magnesium oxide mixed with 25 per cent Celite. When developed with 200 ml. of Skellysolve F, 85 per cent of the material having an absorption maximum at 351 $m\mu$ was washed through the column. When 0.2 gm. of the unsaponifiable portion (representing about 3.0 gm. of the original oil) was diluted to 1 gm. with Wesson oil and treated in an identical manner, the material with an absorption maximum at 351 $m\mu$ was found to be very strongly adsorbed near the top of the column.

Solvent Partition—The adsorption experiments indicated that the rehydro vitamin A was stored in the rat liver as an ester, and was strongly adsorbed after saponification because of the free hydroxyl group. This was confirmed by solvent partition experiments, which were carried out in the following manner: Skellysolve F and 83 per cent aqueous ethanol were shaken together until they were mutually saturated at room temperature. 1.0 gm. of the whole oil and 0.2 gm. of the unsaponifiable portion were dissolved in separate 25 ml. portions of the Skellysolve. Each solution was then extracted seven times with 25 ml. portions of the 83 per cent ethanol. The extinction at 351 $m\mu$ for each extract and for each of the residual petroleum ether solutions was determined on a Beckman spectrophotometer. It was found that the petroleum ether-83 per cent ethanol distribution ratio for the unsaponified material was 98:2, while that for the saponified portion was 45:55. These are approximately the ratios found for vitamin A fatty acid esters and free vitamin A alcohol, respectively.

Attempted Dehydration of Rehydro Vitamin A—Some of the saponified material was dissolved in 0.1 N anhydrous alcoholic hydrogen chloride and allowed to stand for 30 minutes. No change in the ultraviolet absorption spectrum was observed, nor was there any change in the solvent distribution ratio.

Biological Potency of Rehydro Vitamin A—Previous work has shown that vitamin A alcohol, vitamin A esters, and anhydro vitamin A produce the same intensity of blue color with antimony trichloride on a molecular basis. It was assumed that the antimony trichloride blue product of rehydro vitamin A has about the same molecular extinction coefficient as have these above derivatives of vitamin A.

A preliminary biological assay on three vitamin A-depleted rats over a 3 week feeding period indicated that, on the basis of blue color, the new compound possessed approximately one-tenth the activity of vitamin A

and 25 times the activity of anhydro vitamin A. A confirmatory assay on six additional animals was carried out after preparation of a second batch of the conversion product as follows:

90 vitamin A-deficient rats were fed 1.25 mg. daily of crystalline anhydro vitamin A for a period of 32 days. At the end of this time the animals had grown an average of 115 gm. and were sacrificed. The livers were removed, minced, and extracted repeatedly with ethyl ether and alcohol. Most of the phospholipides were precipitated with acetone and the remaining oil (8.2 gm.) was saponified. After removal of sterols, the unsaponifiable portion was dissolved in 3 gm. of Wesson oil for bioassay. The ultraviolet absorption maximum of this preparation was at $351\text{ m}\mu$ with $E_{1\text{ cm.}}^{1\%} = 2.3$. The apparent vitamin A potency by blue color was 2500 units per gm.

This solution was fed to six vitamin A-deficient weanling rats at three levels, two rats per level. The three levels were 77, 44, and 22 apparent blue color units of vitamin A per day.

The two animals on the highest level had gained over 50 gm. apiece at the end of 12 days and feeding was discontinued. At the end of 28 days, the two rats on the middle level had gained an average of 54 gm. apiece and the pair on the lowest level had gained an average of 20 gm. each. By comparison with other vitamin A assays in progress in these laboratories at this time, it was calculated that the lower level was equivalent to approximately 1.5 units and the middle level to approximately 3 units of vitamin A per day. Thus, despite the small number of animals used, these results essentially confirm those of the preliminary assay, indicating that the biological potency of the *in vivo* conversion product, rehydro vitamin A, is about one-fifteenth that of vitamin A itself, but is 15 to 20 times greater than that of the original anhydro vitamin A.

DISCUSSION

The behavior of this new compound with regard to its relative adsorption affinity and solvent distribution before and after saponification indicates that it contains a hydroxyl group and is stored in the liver as a fatty acid ester. The ultraviolet absorption spectrum exhibits a triple peak similar to anhydro vitamin A but the position of these maxima shows that one double bond of the molecule of anhydro vitamin A has been lost. As further confirmation of the double bond structure of rehydro vitamin A, it can be compared to the synthetic hydrocarbons containing five conjugated double bonds, previously synthesized by the author (6). The ultraviolet spectra are almost identical. Since a double bond system in conjugation with a hydroxyl group, as in vitamin A, usually exhibits only a single absorption maximum, the hydroxyl group of rehydro vitamin A is probably isolated from the unsaturated portion of the molecule.

The failure of rehydro vitamin A to be dehydrated in 0.1 N anhydrous alcoholic hydrogen chloride is further evidence of its structure, since vitamin A and many similar compounds readily lose a molecule of water under these conditions. The stability of this substance to a mild anhydrous acid solution would indicate that the ionization postulated by Meunier (2) as a part of the vitamin A dehydration mechanism does not occur in this case, probably because the hydroxyl group is not activated by conjugation with an unsaturated system. Another possible requirement is that the hydroxyl group should be able to undergo an allylic shift to a tertiary carbon atom before dehydration can take place. In either case, these conditions do not appear to be present in the molecule of rehydro vitamin A, indicating that it is probably a primary alcohol whose hydroxyl group is isolated from the double bond system.

On the other hand, antimony trichloride appears to be a strong enough reagent to bring the double bond system and the hydroxyl group into conjugation, thus producing a blue color in the same region as given by vitamin A and anhydro vitamin A. Antimony trichloride has been shown to have this effect on other compounds containing two isolated chromophoric groups,¹ and a similar shift of double bonds may account for the blue-green color produced by the action of antimony trichloride on vitamin A₂ and anhydro vitamin A₂ (7). Thus the structure of rehydro vitamin A given earlier in this paper is proposed as an interpretation of the above experimental findings.

SUMMARY

The growth-promoting activity in the rat of the hydrocarbon anhydro vitamin A has been shown to be due to a new compound formed *in vivo*. A small amount of this substance is stored in the liver as an ester. When extracted from the liver and fed to other vitamin A-deficient rats, this compound has been found to be about 20 times as active as the original anhydro vitamin A, although only about one-fifteenth as active as vitamin A. From a study of its properties, a tentative formula and the name "rehydro vitamin A" have been proposed.

Anhydro vitamin A is toxic to rats at about the same level as vitamin A. The symptoms of toxicity, however, are somewhat different. Doses of 5 mg. per day induce hemorrhage, especially in the testes and epididymides of male rats.

An easy method of preparing anhydro vitamin A in large amounts has been described.

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¹ Shantz, E. M., and Chechak, A. J., unpublished work.

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BIBLIOGRAPHY

1. Shantz, E. M., Cawley, J. D., and Embree, N. D., *J. Am. Chem. Soc.*, **65**, 901 (1943).
2. Meunier, P., Dulou, R., and Vinet, A., *Bull. Soc. chim. biol.*, **25**, 371 (1943).
3. Embree, N. D., *J. Biol. Chem.*, **128**, 187 (1939).
4. Edisbury, J. R., Gillam, A. E., Heilbron, I. M., and Morton, R. A., *Biochem. J.*, **26**, 1164 (1932).
5. Vedder, E. B., and Rosenberg, C., *J. Nutr.*, **16**, 57 (1938).
6. Shantz, E. M., *J. Am. Chem. Soc.*, **68**, 2553 (1946).
7. Shantz, E. M., *Science*, **108**, 417 (1948).

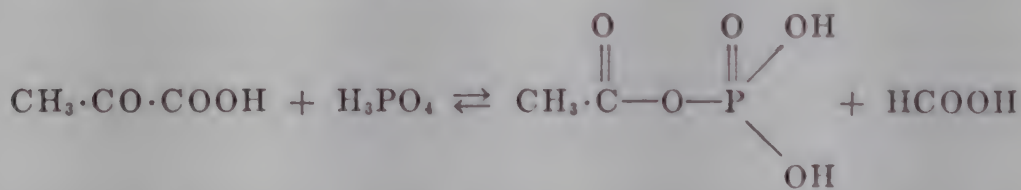
FIXATION OF FORMIC ACID IN PYRUVATE BY A REACTION NOT UTILIZING ACETYL PHOSPHATE*

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Attention was first directed to acetyl phosphate as a possible 2-carbon intermediate in metabolic reactions by Lipmann (1, 2), who isolated the silver salt from the oxidation products formed by lactic acid bacteria from pyruvate. Anaerobically acetyl phosphate was shown to be formed during the dissimilation of pyruvic acid by cell-free extracts of *Clostridium butylicum* (3) and by *Escherichia coli* (Utter and Werkman (4)). In the latter case the reaction was postulated to be



The conversion is referred to as the phosphoroclastic reaction. In further experiments with the cell-free *E. coli* preparation, Utter, Lipmann, and Werkman (5), using C¹³-formate and normal pyruvate, found that the residual pyruvate contained C¹³ in the carboxyl carbon. Likewise carbonyl-labeled pyruvate was formed when carboxyl-labeled acetate, adenosine triphosphate, and normal pyruvate were incubated together. In this latter experiment the incorporation of the isotopic carbon was about only one-twentieth that obtained with C¹³-formate. It was suggested that the slow rate of incorporation of C¹³ from acetate resulted because the formation of acetyl phosphate from acetate was the limiting reaction.

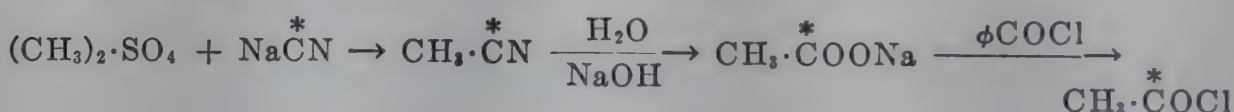
It has generally been considered that the energy-rich phosphate bond of acetyl phosphate made possible the postulated rapid reversible reaction with formate (5). Nevertheless, the evidence for this reaction was indirect, since only formate and acetate were labeled and no direct tests were made with labeled acetyl phosphate. In the present investigation experiments were undertaken with labeled acetyl phosphate to test this point directly.

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Methods

Enzyme Preparation—For the first experiment *E. coli* E-26 was grown in media containing 1 per cent glucose, 0.4 per cent yeast extract, 0.8 per cent dipotassium phosphate, and 10 per cent tap water. For the other experiments the cells were grown in media containing 0.4 per cent beef extract, 0.4 per cent peptone, 0.2 per cent yeast extract, 0.2 per cent NaCl, and 10 per cent tap water. The cells grown in glucose were not aerated; those grown in beef extract and peptone were aerated continuously. The cells were harvested with a Sharples centrifuge after 16 to 18 hours growth at 37°, ground with glass by the method of Kalnitsky *et al.* (6), and extracted with distilled water. Extracts¹ prepared in this way had very little hydrogenlyase activity and did not fix CO₂ into pyruvate under conditions here used, as tested with NaHC¹⁴O₃. The experiments were conducted anaerobically in manometric flasks of 125 ml. capacity at 30.4°. The non-isotopic pyruvate was prepared by neutralizing freshly distilled pyruvic acid with NaHCO₃, and its molarity was calculated on the basis of the decarboxylation with yeast.

Isotopic Compounds—Acetyl phosphate was synthesized from acetyl chloride by the method of Lipmann and Tuttle (7), the acetyl chloride being prepared by the following series of reactions.



The formic acid was prepared by reduction of NaHCO₃, according to the method of Melville *et al.* (8). C¹³H₃·COOH was prepared from C¹³H₃I by the Grignard reaction and CH₃·C¹⁴O·COOH by a method similar to that used by Anker (9). The concentration of the latter was determined by decarboxylation with yeast.

Separation and Degradation of Products—When the acetyl phosphate was to be analyzed for isotope, it was separated from pyruvate, acetate, formate, and other acid products by ether extraction at pH 2.2, thus taking advantage of the fact that acetyl phosphate is a relatively strong acid (pK = 1.1 (10)). The fermentation mixture was cooled in ice water, deproteinized by acidifying with sulfuric acid, and centrifuged. The solution was saturated with anhydrous MgSO₄ (25 gm. per 50 ml. of solution). The pH of the solution was then adjusted to 2.2 and was extracted five times for 2 minutes with 2 volumes of ether. The pH was readjusted to 2.2 with H₂SO₄ after each extraction. The aqueous residue was then steam-distilled, the acetyl phosphate thus being hydrolyzed to acetic acid and recovered as such. The distillate was tested for pyruvate with the nitroprusside reaction (11) and was usually negative, indicating that the separation of pyru-

vate in the extract was quite complete. As a measure of the extent of the separation of the acetyl phosphate from the acetate, the distillate was oxidized with HgO for determination of formate (12). (Formic acid is less soluble in the ether and is a stronger acid than acetic acid.) Only a negligible amount of formate was present, so contamination of the acetyl phosphate with acetate probably was slight. The acetate from the acetyl phosphate was recovered from the residue of the HgO oxidation by steam distillation and was oxidized with persulfate (13) to CO_2 for isotope analysis. The test for pyruvate was always negative in this latter distillate.

The pyruvic, acetic, and formic acids present in the ether extract were extracted with dilute alkali and the pyruvate was removed through conversion to acetaldehyde and CO_2 by incubation with a washed dried yeast at pH 6.0 with a phosphate buffer. The yeast was used in 1.0 per cent concentration. It contained an active carboxylase and produced very little endogenous CO_2 . The acetaldehyde and CO_2 were collected by passing nitrogen gas through the yeast fermentation mixture, and via a reflux condenser into a bead tower containing bisulfite where the aldehyde was trapped. The CO_2 was collected in alkali. At the conclusion of the incubation ($\frac{3}{4}$ hour) the mixture was acidified and boiled to complete the removal of CO_2 and aldehyde. The CO_2 was analyzed for isotope as representative of the carboxyl of the pyruvate. In some experiments the aldehyde originating from the α, β position of the pyruvate was degraded by the iodoform reaction (14) and the resulting formate was converted to CO_2 by HgO oxidation, yielding the α -carbon of the pyruvate; the CHI_3 was oxidized to CO_2 by chromic acid to obtain the β -carbon. In other experiments the aldehyde was oxidized with persulfate to CO_2 to give the combined α - and β -carbons.

After centrifugation of the yeast fermentation, acetate and formate were recovered by steam distillation. The formate was oxidized to CO_2 with HgO and the acetate in the residue of oxidation was recovered by steam distillation. It was oxidized to CO_2 with persulfate (13). The acetate as recovered is in part from acetyl phosphate, which breaks down to acetate during the fermentation and during the separation of products by ether extraction at pH 2.2.

Tests with known mixtures showed that the above method gave satisfactory separation.

In a number of the fermentations in which only the isotope distribution in the residual pyruvate was studied, the reaction mixture was deproteinated by acidification with sulfuric acid. The solution was then adjusted to pH 6.0 and the pyruvate was degraded by yeast fermentation, as described above.

Isotope Concentrations—The C^{13} content of the samples was determined

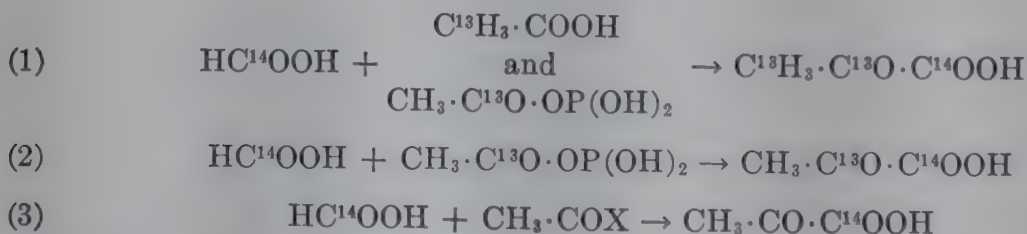
with a Nier-type mass spectrometer (15) and expressed as atom per cent excess C^{13} . The C^{14} was determined by plating as $BaCO_3$ on a sintered glass disk and measuring the activity with a Geiger-Müller window counter, and after correction for background and self-absorption was expressed as counts per minute per mg. of $BaCO_3$ or as counts per minute per mm of compound.

Results

Non-Participation of Synthetic Acetyl Phosphate in Fixation of Formate—In these experiments labeled compounds were added to the enzyme preparation and the fermentation was allowed to proceed to partial completion to permit exchange reactions between the normal and labeled compounds. The residual pyruvate and other products were then separated and degraded for isotope analysis. The reaction mixture consisted of the enzyme, normal pyruvate, $C^{13}H_3 \cdot COOH$, $CH_3C^{13}O \cdot PO_3H_2$, and $HC^{14}OOH$.

Under the experimental conditions used $CH_3 \cdot CO \cdot OPO(OH)_2$ is broken down to $CH_3 \cdot COOH$ plus H_3PO_4 both enzymatically and spontaneously. It was necessary therefore to have an independent measure of the amount of incorporation of acetate in pyruvate. For this purpose, methyl-labeled acetate was added to the reaction mixture.

The distribution pattern of the isotope in the pyruvate would indicate which of the following reactions accounted for the formation of pyruvate from formate.



If acetate were converted to acetyl phosphate which entered pyruvate together with the formate, Type 1 pyruvate would be formed. If acetate did not react but acetyl phosphate were active, Type 2 pyruvate would result. If neither acetyl phosphate nor acetate was an intermediate, but some unknown compound designated as $CH_3 \cdot COX$ was formed from pyruvate and reacted with formate, Type 3 pyruvate would be formed.

It is clear from the results of Table I that only Type 3 pyruvate was formed; there was no excess of C^{13} in the α or β position, whereas the carboxyl group contained a high count which came from formate. These results indicate that synthetic acetyl phosphate is not an intermediate in the fixation of formate in pyruvate, and under the conditions of these experiments acetate was not converted to an intermediate which entered pyruvate.

The isotope results on the acetate, acetyl phosphate, and formate show that there was a much greater dilution of the isotope in the final formate than there was in the acetate or acetyl phosphate. In Experiment I, the isotope in the final formate was diluted about 57 per cent, whereas the acetate was diluted only 14 per cent and the isotope of acetyl phosphate 18 per cent. In Experiment II, the formate was diluted 34 per cent and the acetate 13 per cent. A high dilution of the formate would be expected if it took part in a reversible exchange with the carboxyl of the pyruvate, whereas the acetate and acetyl phosphate would be diluted less, since the only dilution which would occur in these latter compounds (if they did not

TABLE I

Formate Incorporation in Pyruvate As Compared to Acetyl Phosphate and Acetate

Enzyme preparation No.*	Final pyruvate			HC ¹³ OOH		C ¹³ H ₃ -COOH		CH ₃ -C ¹³ O-PO ₃ H ₂	
	CH ₃ —	—C=O	—COOH	Added	Final	Added	Final	Added	Final
	per cent C ¹³	per cent C ¹³	c.p.m. per mM	c.p.m. per mM	c.p.m. per mM	per cent C ¹³	per cent C ¹³	per cent C ¹³	per cent C ¹³
I	0.01	—0.01	3950	12,000	5240	2.07	1.79	2.03	1.66
II	—0.02	—0.01	2540	12,000	8050	2.07	1.80	2.03	

A separation and degradation of pyruvate carried out on a mixture at zero time showed that there was no incorporation of isotope in the pyruvate during the fractionation. The original reaction mixture contained the following materials in 30 ml.: pyruvate 2.0 mM, no isotope; formate 1.9 mM, 12,000 counts per minute per mM; acetyl phosphate 2.2 mM, 4.06 per cent C¹³ excess in carboxyl carbon; acetate 1.5 mM, 4.14 per cent C¹³ excess in methyl carbon; enzyme 12 ml.; and phosphate buffer (pH 6.8) 2.25 mM. Gas phase was 5 per cent CO₂-95 per cent N₂. The isotopic compounds and pyruvate were tipped in from the side arm after equilibration. The incubation was at 30.4° for 3 hours.

* There was no C¹⁴ in fractions for which C¹³ determinations are given in Table I, and likewise no C¹³ in the fraction containing C¹⁴.

exchange) would be the amount formed as end-products from pyruvate metabolism. All these results indicate that formate enters pyruvate by combination with some compound other than synthetic acetyl phosphate or acetate. The methods used were designed primarily for the separation of fractions of the products for isotope analysis rather than for quantitative recovery of the products, so that no direct measurement of the yield of products was obtained; but it is clear from the isotope dilution that the amount of conversion of pyruvate to acetate or acetyl phosphate was not great. This point will be discussed in greater detail later in the paper.

Non-Participation of Biological "Acetyl Phosphate" in Fixation of Formate—Experiments next were conducted to determine whether a product of pyruvate metabolism was a reactive intermediate in this system. Lipmann

(16) has developed a colorimetric determination for acetyl phosphate using hydroxylamine and FeCl_3 . The method is not specific since any acid anhydride will give the reaction. When the *E. coli* enzyme metabolizes pyruvate, a product is formed which gives this reaction and, calculated on the basis of acetyl phosphate, as much as 50 per cent of the fermented pyruvate is accountable as accumulated phosphate anhydride in some fermentations if NaF is included to prevent the breakdown of the anhydride.¹ It seemed possible that the product might be the reactive intermediate which combined with formate but differed in some unknown manner from synthetic acetyl phosphate.

An experiment was set up as follows to test this point and consisted of three parts: (1) Fermentation of the C^{14} -pyruvate to obtain a C^{14} -biological compound with a high specific activity. The original pyruvate had a specific activity of 400,000 counts per minute per mm, as is shown under Step I, Table II. For purposes of illustration the reaction may be written



$\text{CH}_3 \cdot \text{COX}$ is used in the above equation and elsewhere for the purpose of indicating the uncertainty of the identity of the 2-carbon compound.

(2) The second step consisted of dilution of the C^{14} -pyruvate remaining after the first reaction by addition of non-isotopic pyruvate so as to give it a lower specific activity than the biological compound. This dilution would make it possible to detect the reentrance of the C^{14} -biological compound because it would have a greater activity than the diluted pyruvate. At the same time C^{14} -formate was added to permit measurement of the fixation of formate. In addition normal lactate and succinate were added to dilute these sources of high activity carbon. An aliquot of the mixture was re-

¹ Considerable difficulty was encountered in obtaining an enzyme preparation which gave a strong phosphoroclastic reaction such as observed by Utter *et al.* (5), who found 85 per cent of the pyruvate fermented to formate and acetate. Many enzymes prepared from *E. coli* 26, following their method, produced almost entirely a dismutation reaction to lactate, acetate, and CO_2 , although the preparation used in Experiment I did give a considerable yield of formate. Better formate production was obtained by using growth media containing peptone, beef extract, yeast extract, and NaCl with continuous aeration (6). The extract obtained by glass grinding produced mostly acetate and formate with smaller amounts of lactate and succinate. Phosphatase activity was quite high, however, and very little acetyl phosphate accumulated unless NaF was present. Addition of NaF was not necessary with preparations obtained by Utter's technique. Studies of preparations made by disrupting the cells with a supersonic oscillator were also made. These preparations, while very active, had two disadvantages. Phosphatase activity was so high that NaF had little effect and the preparation lost activity in a few days even when maintained frozen.

moved for determination of the amount of pyruvate and determination of the C^{14} in the pyruvate and formate. The hydroxylamine reaction was also measured and the amount of $CH_3 \cdot COX$ was calculated. The results from these analyses are shown under Step II, Table II. (3) The aliquot remaining after the removal of the above sample was submitted to a second fermentation of $1\frac{1}{2}$ hours. This fermentation permitted exchange in the pyruvate of the C^{14} -formate and of the C^{14} -biological compounds. The $CH_3 \cdot COX$ is shown in Table II, but C^{14} -acetate is formed as well which was not diluted with normal acid in Step II. The changes in the C^{14} distribution were determined by analyses of the C^{14} in the pyruvate and formate. The results are shown under Step III, Table II.

The protocol follows:

Step I— $CH_3 \cdot C^{14}O \cdot COONa$ 0.84 mM (400,000 c.p.m. per mM), 0.5 M phosphate buffer (pH 6.8) 6 ml., 0.6 M NaF 3 ml., enzyme extract 8 ml., H_2O to make up to 19.0 ml. total volume, and atmosphere nitrogen; incubation 80 minutes at 30.4° .

Step II—Reaction mixture from Step I 18.1 ml., 0.50 M lactate 1.5 ml., 0.50 M succinate 0.6 ml., 0.43 M pyruvate 6.2 ml., 1.4 M formate (200,000 c.p.m. per mM) 0.14 ml., and enzyme extract 8.0 ml. The total volume was 34.6 ml. and the solutions added were ice-cold. 15.4 ml. were removed for hydroxylamine reaction and for determination of C^{14} in the pyruvate and formate.

Step III—19.2 ml. of mixture from Step II were incubated $1\frac{1}{2}$ hours at 30.4° under nitrogen and then the pyruvate was degraded.

The amounts of normal lactate and succinate added in Step II were calculated to dilute the acids formed in Step I by 10-fold, *i.e.* approximately the same dilution as the pyruvate. The basis for the calculation was an experiment run previously in which the amounts of pyruvate metabolized and products formed were determined.² The amount of formate added was calculated to contain approximately the same total activity as the $CH_3 \cdot COX$ present at the end of Step I. Unless the lactate and succinate were diluted to the same extent as, or a greater extent than, the pyruvate, any increase in the specific activity of pyruvate in Step III could conceivably come from these acids reforming pyruvate, just as from $CH_3 \cdot COX$ or acetate.

² The fermentation was set up as follows: enzyme 12.0 ml., 0.6 M NaF 3 ml., 0.5 M phosphate buffer (pH 7.0) 3 ml., 0.45 M pyruvate 3 ml., H_2O 6 ml. Incubation 5 hours at 30.4° under N_2 gas. CO_2 determined manometrically with acidification at the conclusion. The 1.35 mM of pyruvate was completely fermented. The products were as follows: CO_2 = 0.34 mM, formate = 0.66 mM, acetate = 1.10 mM, lactate = 0.20 mM, succinate = 0.08 mM. Carbon recovery = 101.7 per cent. Oxidation-reduction balance = 104.7. It was assumed from preliminary experiments that approximately 0.3 mM of pyruvate would be fermented in Step I.

It is seen under Step II that after the initial fermentation and addition of non-isotopic pyruvate, lactate, succinate, and C^{14} -formate the α,β -carbons of the pyruvate had a specific activity of 44,500 counts. The formate had 50,000 counts and presumably the biological compound designated as $CH_3 \cdot COX$ would have a maximum activity of 400,000 counts. However, there may be some dilution, due to endogenous $CH_3 \cdot COX$ or exchange reactions. For this reason a separate experiment was run in which carbonyl-

TABLE II

Formate Exchange As Compared to That of Biological Product from Pyruvate

Substances*	Step I, prior to fermentation	Step II, initial analysis after 1st fermentation and additions	Step III, analysis after 2nd fermentation
$CH_3 \cdot CO \cdot COOH$, mm.....	0.45	1.60	1.08
α,β -Carbons, c.p.m. per mm.....	400,000	44,500	40,500
Carboxyl, c.p.m. per mm.....		119	7,480
$CH_3 \cdot COX$, mm.....		0.12†	0.08
C.p.m. per mm.....		300,000‡	
$HCOOH$, mm.....		0.37	0.48
C.p.m. per mm.....		50,000	8,140

* The mm quantities for all steps are calculated on the basis of the final portion used in Step III.

† The value for mm of $CH_3 \cdot COX$ is that determined by the hydroxylamine reaction. It is a minimum value, since phosphate and fluoride both decrease the color intensity. In a separate experiment(§), the isolated acetate from the phosphate anhydride was actually twice that determined by the hydroxylamine reaction.

‡ The specific activity of $CH_3 \cdot COX$ was calculated on the basis of a separate experiment in which pyruvate with a specific activity of 1,300,000 counts per minute per mm was fermented by the enzyme extract for 1 hour. The phosphate anhydride formed was then separated and degraded in the same manner as described in Experiment I for acetyl phosphate. The specific activity of the acetate of the anhydride was found to be 970,000 counts per minute per mm.

labeled pyruvate was fermented and the $CH_3 \cdot COX$ was separated from the other acids by ether extraction at pH 2.0, as described for acetyl phosphate separation in Experiment I. The dilution of the $CH_3 \cdot COX$ was found to be 25 per cent, and therefore the count of the $CH_3 \cdot COX$ was listed as 300,000. After these materials were permitted to equilibrate, it is seen that the α,β -carbons did not increase in specific activity; actually they decreased from 44,500 counts to 40,500. The carboxyl carbon on the other hand increased from 119 to 7480 counts, while the specific activity of the formate dropped from 50,500 to 8140. These latter results are what would be expected if the non-isotopic carboxyl carbon of the pyruvate came to

approximate equilibrium with the C^{14} -formate. Since the pyruvate and lactate were probably in reversible equilibrium through a lactic dehydrogenase action, a substantial amount of the formate isotope would enter the lactate. Also in breakdown of the pyruvate some C^{14} would enter the CO_2 . Thus only part of the C^{14} of the added formate would be accounted for in the products analyzed.

The fact that there was a decrease in the specific activity of the α,β -carbons of the pyruvate after the second reaction (40,500 as compared to 44,500 counts) rather than an increase may have been caused by an excessive dilution of the lactate and succinate in Step II. Thus, if these acids were diluted more than the pyruvate, they would serve to dilute the pyruvate activity during the course of the Step III fermentation.

As a further test of this point, another experiment was conducted and the results are given in Table III. Here Step I was set up in a similar manner to Step I of the preceding experiment, except that the original pyruvate had a specific activity of 1,300,000 counts per minute per mM and presumably $CH_3 \cdot COX$ formed would have a very high specific activity, which has been listed in Table III as being 970,000 counts per minute per mM, as found in the separate experiment. Step II consisted of chilling the mixture in an ice bath and separation into two approximately equal portions.

Portion 1 was treated in the same way as in the previous experiment; *i.e.*, the C^{14} -pyruvate was diluted with normal pyruvate and C^{14} -formate was added, but there was no addition of normal lactate or succinate. An aliquot was removed and degraded for determination of the isotope content of the pyruvate and formate at Step II, and the remainder was incubated with more enzyme and was then degraded at the end of Step III.

Portion 2 was used as a control for Portion 1, in that the C^{14} -labeled acid anhydride was destroyed prior to Step II and the incubation of Step III. This was accomplished by incubating Portion 2 at 40° for 40 minutes. Acetyl phosphate is unstable at neutral pH at that temperature. The hydroxylamine reaction was followed and the incubation was continued until the reaction was negative. Thus the anhydride which had been formed from the C^{14} -pyruvate was destroyed. The normal pyruvate and C^{14} -formate were then added and an aliquot was then withdrawn for pyruvate and formate degradation and determination of isotope content at Step II. The remainder of Portion 2 was then incubated at 30° similarly to the remainder of Portion 1. By this procedure aliquots of the same fermentation could be compared, one of which contained an anhydride with a high specific activity and the other no anhydride. If the anhydride did combine with formate to form pyruvate, then Portion 1 should show an increase of isotope concentration in the carbonyl group of pyruvate much higher than Portion 2, in which any increase in this carbonyl isotope con-

centration could be derived only from the products acetate, lactate, and succinate.

The protocol follows:

Step I— $\text{CH}_3 \cdot \text{C}^{14}\text{O} \cdot \text{COOH}$ 0.8 mM (1,300,000 counts per minute per mM), 0.5 M phosphate buffer (pH 6.8) 7.0 ml., 0.6 M NaF 4.4 ml., enzyme extract 18.0 ml., H_2O to make to 51.4 ml., and atmosphere nitrogen; incubation $1\frac{1}{2}$ hours at 30° .

Step II—Reaction mixture from Step I cooled in ice and divided into Portions 1 and 2. Portion 2 incubated 40 minutes at 40° until hydroxylamine reaction was negative. Portions 1 and 2 were simultaneously set up as follows: reaction mixture from Portion 1 or 2, 23.5 ml., 0.40 M pyruvate 6.0 ml., 0.3 M formate (110,000 c.p.m. per mM) 5.0 ml., 0.6 M NaF 1.1 ml.,

TABLE III

Formate Exchange As Compared to That of Biological Products from Pyruvate
The values are given in counts per minute per mM.

Substance	Portion 1 Acid anhydride present		Portion 2 Acid anhydride destroyed	
	Step II	Step III	Step II	Step III
Carboxyl of pyruvate.....	765	36,500	604	32,600
α, β -Carbons of pyruvate.....	5,740	9,120	4,480	7,880
$\text{CH}_3 \cdot \text{COX}$	970,000*			
HCOOH	102,000		102,000	44,000

* See the note (§) under Table II. The amount determined by the hydroxylamine reaction was found to be 0.13 mM. However, as noted under Table II, the actual amount was probably twice this.

0.5 M phosphate buffer (pH 6.8) 1.7 ml., enzyme extract 5.0 ml., and total volume 42.3 ml. 20 ml. were removed for degradation at Step II.

Step III—22.3 ml. of mixture from Step II incubated $1\frac{1}{2}$ hours at 30.4° under nitrogen and then pyruvate was degraded.

In Table III it will be observed that the increase in specific activity of the α - and β -carbons of the pyruvate is almost the same in both portions. The increase in Portion 1 was 3380 and, in Portion 2, 3400. Since in this experiment there was no dilution of the formed acetate, lactate, and succinate, as in the previous experiment, if these compounds reformed pyruvate, they would cause an increase in the specific activity of the residual pyruvate. From these sources the increase should be approximately the same in both portions, since acetate, lactate, and succinate are end-products and were present in approximately equal concentration. This is probably the case, since there is little fermentation during the incubation at 40° and the pyruvate was almost completely fermented during the first incubation. That

there was some fermentation, however, is indicated by the lower specific activity in the pyruvate (Table III) in the fermentation in which the "acetyl phosphate" was destroyed.

Thus it is observed from two experiments with labeled pyruvate that a metabolic product accumulates which reacts with hydroxylamine under the conditions of Lipmann's test for acyl phosphates (16). This compound, however, does not appear to be a necessary intermediate in the fixation of formic acid in pyruvate. A further corollary is that other products of pyruvate metabolism such as acetate do not take part in the synthesis, since any such compound would presumably contain isotope and would therefore increase the isotope level in the carbonyl group of the final pyruvate, just as the carboxyl group isotope concentration was increased by the added isotopic formate, but no such extensive increase was observed.

Lack of Correlation between Phosphoroclastic Reaction and Fixation of Formate—In order to obtain further information on the mechanism of formic acid fixation a study of the enzyme systems involved has been initiated. It was early learned that allowing the usual cell-free extract to stand unfrozen at 0 to 2° for 2 days would usually cause almost all of the phosphoroclastic activity to disappear. The phosphoroclastic activity could be roughly approximated by determining the CO₂ formation in NaHCO₃ buffer, since the formation of the two acids, acetate and formate, from pyruvate would result in formation of one excess acid per pyruvate fermented. The "aged" preparation which had lost most of its activity, as measured by CO₂ formation in bicarbonate, continued to fix formate at about the same rate as an "unaged" preparation. Table IV presents some representative data.

The experiments were conducted as follows: After incubation at 30.4° for 1 hour under 60 per cent CO₂ and 40 per cent nitrogen, the mixture was deproteinated with 3 N H₂SO₄. The proteins were removed by centrifugation and the supernatant solution was placed in a Warburg flask and gassed with CO₂ and then nitrogen while shaking. This procedure removed all the C¹⁴O₂ from the mixture. 2.5 N NaOH was then introduced into the center well of the Warburg flask and ceric sulfate into the side arm. The ceric sulfate was tipped into the main compartment and oxidized the residual pyruvate to acetate and CO₂. The CO₂ was trapped in the alkali and plated as BaCO₃ for C¹⁴ determination. Decarboxylation by yeast gave similar results.

From the experiments shown in Table IV it is clear that, although there was a large loss in enzyme activity in forming acid products, there was no loss in fixing formate. This conclusion has been checked by direct determination of the acetate and formate produced from pyruvate by using a number of enzyme preparations.

Closely related to the findings with the enzyme extract for *E. coli* is the

observation that *Staphylococcus aureus* will fix formate in pyruvate quite rapidly. This fixation was rather unexpected because the principal reaction produced by these bacteria is a dismutation reaction



and very little formate is formed as an end-product.

In the experiment shown in Table V the pyruvate was allowed to be completely fermented and C^{14} was determined in the end-products. It is seen that there was fixation of formate in the carboxyl of lactate and some isotope occurred in the metabolic CO_2 . The lactate is considered to have been in equilibrium with the pyruvate and probably in this way acquired the isotope. That there was fixation in the pyruvate was shown in experiments of shorter duration in which the pyruvate was not completely fer-

TABLE IV
Comparison of Rate of Acid Production and Rate of Fixation of Formate

Preparation	CO ₂ from NaHCO ₃	COOH of pyruvate	
		Total activity	BaCO ₃
	$\mu\text{l.}$	<i>c.p.m.</i>	<i>c.p.m. per mg.</i>
Unaged	422	498	28.1
Aged 24 hrs.	240	479	26.9
" 48 "	63	433	27.3

Additions: enzyme extract 0.8 ml., $\text{CH}_3\cdot\text{CO}\cdot\text{COONa}$ 0.1 mM, HC^*OONa 0.1 mM (10,000 c.p.m. per mM), phosphate buffer, pH 6.8, 0.1 mM, NaHCO_3 0.05 mM, and H_2O to make 2 ml.

mented. In these experiments a similar amount of fixation occurred in the carboxyl of the pyruvate. This fact was shown by decarboxylation with yeast; also the residual pyruvate was isolated as the 2,4-dinitrophenylhydrazone and shown to be active.

It is clear from the experiment of Table V that the principal reaction is a dismutation reaction with acetate, CO_2 , and lactate as products. Although the results of Table V indicate that there was no net production of formate, but rather a small disappearance, in control experiments to which no formate was added, it was possible to detect a very small production of formate from pyruvate. Approximately 1 to 3 mM per 100 mM of fermented pyruvate were formed. The formate was identified by action of hydrogenlyase in washed *E. coli* bacteria. In conducting the test the steam distillates were concentrated and hydrogen formation from the distillate was measured in the respirometer. Also the formate was determined by HgO oxidation.

Under the conditions of these experiments the carboxyl of pyruvate did

not come to equilibrium with the formate, having one-sixth the activity of the residual formate. Thus the fixation was not as rapid as with the *E. coli* enzyme. Although this fixation may be accounted for on the basis of reversal of the reaction by which formate is formed, in view of the results with *E. coli*, it seems probable that the fixation may occur by a mechanism not necessarily linked with this reaction. Further study is needed to clarify this point.

TABLE V
Fixation of Formate by Staphylococcus aureus

Compound	Added	Final	C.p.m. per min. per mm
	mm	mm	
Pyruvate.....	5.95	0.0	
Formate.....	4.0	3.75*	13,200
CO ₂		2.81	1,600
Acetate.....		2.43	
Lactate†.....		3.00	2,200

Reaction mixture in each of two flasks: 0.40 M formate (14,140 counts per minute per mm) 5 ml., 0.5 M phosphate buffer (pH 7.0) 10 ml., 0.425 M pyruvate 7 ml., lyophilized *staphylococcus* 1.0 gm.; total volume was 30 ml. (The *staphylococcus* was grown on 0.2 per cent yeast extract, 0.4 per cent peptone, and 0.4 per cent beef extract agar.) Anaerobic with nitrogen; temperature 30.4°; incubation time 70 minutes. Duplicate flasks were set up and one was used for manometric determination of the CO₂, including bound CO₂. To the other alkali was introduced in the center well at the end of the incubation and a sample of CO₂ was collected for isotope analysis. The values are for the combined fermentations of the two flasks and, since the pyruvate was completely fermented, the data represent the products from 5.95 mm.

* Note that 4.0 mm of formate were added; thus there was a breakdown of 0.25 mm of formate.

† Lactate was oxidized to acetaldehyde and CO₂ with KMnO₄ and activity was exclusively in the carboxyl group.

The production of isotopic CO₂ is probably indirect; *i.e.*, by fixation of formate in the pyruvate and then breakdown of the pyruvate. At any rate there is little or no isotope liberated as CO₂ if formate is the only substrate. When NaHC¹⁴O₃ was incubated with pyruvate, there was very little fixation of CO₂ in the pyruvate, so that the CO₂ fixation reaction apparently does not play a major rôle in the results.

DISCUSSION

The experiments reported in this paper demonstrate that it is possible for formic acid to be fixed in the carboxyl group of pyruvic acid without

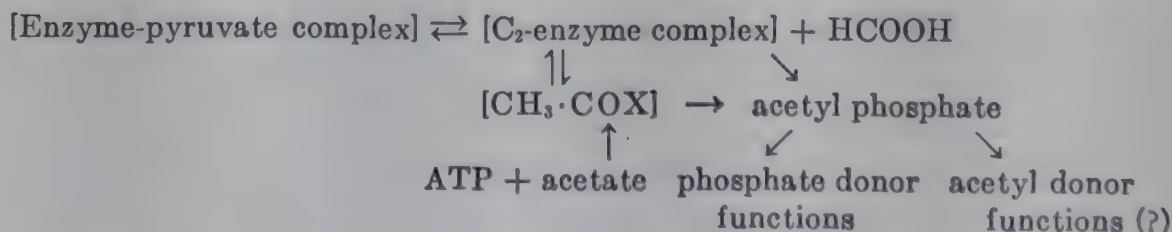
concomitant fixation of acetyl phosphate, acetate, or other accumulated metabolic end-products containing the carbonyl group of pyruvate.

Needless to say the non-participation of acetyl phosphate in the fixation of formate does not detract from its metabolic importance. The results of Stadtman and Barker (17) have indicated an important rôle for acetyl phosphate in fat metabolism and the results of Kaplan and Lipmann (18) indicate that a compound closely related to acetyl phosphate is formed from acetate and adenosine triphosphate (ATP). This latter compound apparently combines with formate to form a keto acid, presumably pyruvate. The biological product of the ATP and acetate reaction on treatment with acid is converted to acetyl phosphate. If this biological product is formed in the breakdown of pyruvate, it appears that it does not accumulate as such under the conditions of our experiments but is converted to a form which is unreactive and is perhaps acetyl phosphate.

An analogy may be made with the finding of Utter and Chenoweth (19) in the fixation of pyruvate and CO_2 in oxalacetate. They found, by use of different enzyme preparations and conditions with labeled pyruvate and CO_2 , that either CO_2 or pyruvate could be fixed independently of the other; however, with proper conditions, both would be fixed. It seems possible that there are active forms of both CO_2 and pyruvate which enter the final step of the fixation and that with proper conditions both of these compounds can be converted to active forms. Viewed in this respect, our preparation might be incapable of converting the acetyl phosphate to an active form, but could form the active anhydride from pyruvate, though not in accumulated quantities which could be detected by the methods used here.

Two possibilities may be suggested as starting hypotheses for explaining our results and possibly correlating them with the results of Kaplan and Lipmann.

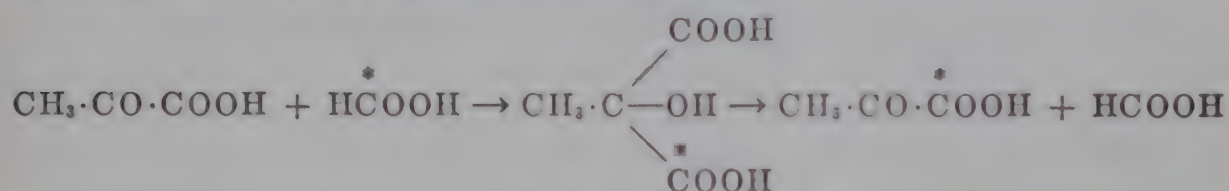
Combination of formate with a precursor of acetyl phosphate, which precursor may be either a reactive compound or an active group forming a complex with the enzyme, the reaction being pictured in this way:



The C_2 -enzyme complex, being in dynamic equilibrium with the enzyme-pyruvate complex, would be able to fix formate. Presumably the anhydride of Kaplan ($\text{CH}_3 \cdot \text{COX}$) could combine with the enzyme and pyruvate could be formed in combination with formate. Also it would be assumed that $\text{CH}_3 \cdot \text{COX}$ did not accumulate in our enzyme preparation but that

acetyl phosphate did. From this point of view the aging of the preparation in experiments presented in Table IV would be considered in some way to inactivate the step, C_2 -enzyme complex \rightarrow acetyl phosphate. In agreement with this suggestion, it should be noted that there is no accumulation of acetyl phosphate as measured by the hydroxylamine method with the aged preparation.

Another possibility is that formate may enter pyruvate without formation of a 2-carbon compound. For example, formate may combine with pyruvate itself or an active form thereof to yield some sort of symmetrical dicarboxylic (or tricarboxylic) acid which, on breaking down again to pyruvate and formate, would exchange isotopic with non-isotopic carbon in the carboxyl groups as illustrated below.



As yet we have no evidence that any such addition compound accumulates in our enzyme system. Experiments are being conducted with variously treated preparations to explore the possibilities of either trapping or accumulating the hypothetical compound, and the enzyme preparations are being fractionated in an effort to learn more about the components of the system.

SUMMARY

Enzyme extracts may be prepared from *Escherichia coli* which rapidly fix formate in the carboxyl group of pyruvate but do not fix either synthetic acetyl phosphate or an acid anhydride formed from pyruvate. It is concluded that acetyl phosphate as such is not a direct intermediate in the fixation of formate in pyruvate. Formate is rapidly fixed by enzyme preparations which produce little or no net breakdown of pyruvate. Furthermore *Staphylococcus aureus* fixes formate in pyruvate, even though it does not produce an appreciable amount of formate. There is thus no correlation between the extent of the so called phosphoroclastic reaction and fixation of formate. Possible explanations are considered.

BIBLIOGRAPHY

1. Lipmann, F., *J. Biol. Chem.*, **155**, 55 (1944).
2. Lipmann, F., *Advances in Enzymol.*, **6**, 231 (1946).
3. Koepsell, H. J., Johnson, M. J., and Meek, J. S., *J. Biol. Chem.*, **154**, 535 (1944).
4. Utter, M. F., and Werkman, C. H., *Arch. Biochem.*, **5**, 413 (1944).
5. Utter, M. F., Lipmann, F., and Werkman, C. H., *J. Biol. Chem.*, **158**, 521 (1945).

6. Kalnitsky, G., Utter, M. F., and Werkman, C. H., *J. Bact.*, **49**, 595 (1945).
7. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **153**, 571 (1944).
8. Melville, D., Rachele, J. R., and Keller, E. B., *J. Biol. Chem.*, **169**, 419 (1947).
9. Anker, H. S., *J. Biol. Chem.*, **176**, 1333 (1948).
10. Lipmann, F., and Tuttle, L. C., *Arch. Biochem.*, **13**, 373 (1947).
11. Simon, L. J., and Piaux, L., *Bull. Soc. chim. biol.*, **6**, 477 (1924).
12. Osburn, O. L., Wood, H. G., and Werkman, C. H., *Ind. and Eng. Chem., Anal. Ed.*, **5**, 247 (1933).
13. Osburn, O. L., and Werkman, C. H., *Ind. and Eng. Chem., Anal. Ed.*, **4**, 421 (1932).
14. Wood, H. G., Werkman, C. H., Hemingway, A., and Nier, A., *Proc. Soc. Exp. Biol. and Med.*, **46**, 313 (1941).
15. Nier, A. O., *Rev. Scient. Instruments*, **2**, 212 (1940).
16. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **159**, 21 (1945).
17. Stadtman, E. R., and Barker, H. A., *J. Biol. Chem.*, **174**, 1039 (1948).
18. Kaplan, N. O., and Lipmann, F., *J. Biol. Chem.*, **176**, 459 (1948).
19. Utter, M. F., and Chenoweth, M. T., *Federation Proc.*, **8**, 261 (1949).

THE AMINO ACID REQUIREMENTS OF MAN

I. THE RÔLE OF VALINE AND METHIONINE*

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With the isolation and identification of threonine (1, 2) it became feasible, for the first time, to support the growth of animals upon diets carrying appropriate mixtures of highly purified amino acids in place of proteins. By the successive removal from such diets of single amino acids, the nutritive rôle of each has been established (3). The results demonstrate that, for the growing rat, ten amino acids are essential dietary components. These are valine, leucine, isoleucine, threonine, methionine, phenylalanine, tryptophan, lysine, histidine, and arginine. The exclusion from the food of any one of these, other than arginine, leads to a profound nutritive failure, loss in weight, diminished appetite, and eventual death. In contrast to these spectacular effects, arginine deprivation merely decreases the rate of gain (4). This is accounted for by the fact that arginine can be synthesized by the rat (5), but not at a rate which keeps pace with the needs of the organism for maximum growth. This behavior distinguishes arginine from the amino acids of the non-essential group. The latter apparently can be manufactured by the cells in sufficient amounts to meet fully the requirements of the animal.

The above experiments provide much information concerning the types of synthetic reactions which the animal organism can and cannot accomplish. Furthermore, the growth technique developed in these studies affords an almost unlimited opportunity of extending this information in so far as it applies to the synthesis of amino acids. By the use of properly formulated diets one can determine the ability of living cells to transform a wide variety of synthetic compounds into the essentials. The results of certain experiments of this nature have already been published (6-8), and others will be presented in subsequent papers.

In the meantime, it is well to bear in mind that findings obtained in the rat are not necessarily transferable to other species, particularly man.

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† The experimental data in this paper are taken from a thesis submitted by Julius E. Johnson in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the University of Illinois.

This fact has been recognized throughout the course of our animal investigations. One of our ultimate objectives has always been the establishment of the amino acid requirements of man. It was anticipated that time and expense might be saved by first conducting the animal tests, and using the data thereby obtained as the starting point in the more difficult and costly studies upon human subjects. Subsequent events have fully sustained this supposition.

Investigations in the human species were first undertaken in this laboratory during the autumn of 1942, and since then have been in progress continually. Publication of the detailed findings has been postponed deliberately in order to permit the accumulation of a large number of data upon a comparatively large number of individuals.¹ It was hoped thereby to diminish the probability of errors in interpretation. Experiments have now been completed upon more than forty individuals. The consistent findings appear to warrant the belief that other human subjects would behave similarly. Thus, we now feel sufficiently sure of the outcome to present our data in full.

The present paper describes the general procedures, analytical methods, and dietary régimes employed throughout this entire series of studies. It also contains evidence for the essential nature of valine and methionine as components of the diet of man. Later papers will demonstrate the qualitative behavior of the other amino acids. Finally, evidence will be presented as to the minimum amount of each essential which is required for the maintenance of nitrogen equilibrium.

EXPERIMENTAL

General Procedures—Throughout the investigations, adult males have served as the experimental subjects. For the most part, these young men have been graduate students in biochemistry, or students in related fields who have had sufficient experience in biochemistry courses to appreciate the significance of the findings. Thus, they had a personal interest in the outcome of the studies.

In view of the wide-spread distribution of proteins and other nitrogenous materials in ordinary foods, the diets were necessarily limited to a very small number of components. These consisted of amino acids, corn-starch, sucrose, purified butter fat, corn oil, cod liver oil, inorganic salts, centrifuged lemon juice, Cellu flour, and an appropriate mixture of vitamins. The amino acids, which always were carefully purified until they yielded

¹ Two preliminary reports of this general program have been published (9, 10). A paper embodying a summary of the qualitative findings was presented by request before a joint meeting of the American Philosophical Society and the National Academy of Sciences in Philadelphia on October 22, 1946 (11).

correct analytical values, furnished approximately 95 per cent of the nitrogen intake. The remaining 5 per cent represented the irreducible amount, of an unknown nature, which was present chiefly in the starch, and, to a much less extent, in the other constituents of the diets. It seems unlikely that this relatively small quantity of unidentified nitrogen could have influenced the findings significantly.

The state of the nitrogen balance served as the criterion of adequacy or inadequacy of the rations. Invariably, the diets were consumed for 2 or 3 days preceding the collection of the excreta in order to permit the subjects to establish nitrogen equilibrium. 24 hour urine samples were then collected each day, diluted to constant volumes, and preserved under toluene until analyzed. At the beginning of each dietary régime the feces of each individual were marked off by the consumption of charcoal tablets. The daily output of fecal material was weighed, mixed thoroughly with alcohol containing a small amount of sulfuric acid, and evaporated to dryness on a steam cone. At the end of the period, the daily quantities were combined, thoroughly ground, and mixed for analysis. The average daily excretion of fecal nitrogen was obtained by dividing the total output by the number of days in the period.

After nitrogen equilibrium had been established, single amino acids were removed from the ration one at a time, while the total nitrogen intake was maintained at a constant level by proportionate increases in the others. Thus, it was possible to demonstrate whether the amino acid which had been excluded from the food was necessary for nitrogen balance.

In all of the experiments designed to establish the qualitative rôle of the amino acids in nitrogen equilibrium, the urines were analyzed not only for total nitrogen, but also for urea, ammonia, creatinine, creatine, uric acid, and α -amino acids. This was for the purpose of determining whether unique alterations in the distribution of nitrogen were associated with specific types of dietary deficiencies. Furthermore, at the end of each period venous blood samples were withdrawn from the arm, and were analyzed for hemoglobin, total plasma proteins, and whole blood non-protein nitrogen. Simultaneously, erythrocyte and leucocyte counts were made.

Methods of Analysis—Total nitrogen was determined in the urines, feces, and components of the diets according to the Scales and Harrison (12) modification of the Kjeldahl procedure. In the earlier experiments, potassium sulfate and copper sulfate served as the catalysts. In all subsequent analyses of the feces and dietary components, including the amino acids, a mixed catalyst composed of potassium sulfate, copper sulfate, and selenium dioxide was employed and the digestions were continued for 8 hours (*cf.* Chibnall *et al.* (13)).

In analyzing the urines, all colorimetric procedures were adapted to the use of the Evelyn photoelectric colorimeter. In each case, the volume of the sample was so adjusted as to provide a satisfactory galvanometric reading. For each compound to be determined, a standard curve was established in the customary manner by treating samples of known purity with the same reagents and under the identical conditions used in the routine analyses.

Urea plus ammonia was determined by a modification of the method of Folin and Youngburg (14). Prior to each analysis, the potency of the urease preparation was tested upon a solution containing a known amount of urea. Ammonia was determined by the procedure of Folin and Bell (15), and the urea was obtained by difference. Creatinine and creatine were estimated by adaptations of the methods of Folin (16). In all of these techniques a 520 m μ filter was used in making the colorimetric comparisons.

For the determination of uric acid a modification of the procedure of Benedict and Franke (17) was employed, urea being added to prevent the development of turbid solutions (*cf.* Christman and Ravwitch (18)). A 600 m μ filter was used in making the colorimetric comparisons. The free amino acids of the urines were estimated by the gasometric-ninhydrin method of Van Slyke, MacFadyen, and Hamilton (19).²

Whole blood and plasma samples were analyzed for non-protein nitrogen by a modified Koch-McMeekin procedure (20) in which a 420 m μ filter was used. Total nitrogen of the plasma was estimated by the method of Koch (21). The difference between the total and non-protein nitrogen of the plasma, multiplied by 6.25, was taken as the level of total plasma proteins. Hemoglobin was measured according to the procedure of Sanford, Sheard, and Osterberg (22). Blood cell counts were carried out in the customary fashion. Hayem's and Turk's solutions were used as the diluents for the erythrocytes and leucocytes respectively.

Composition of Diets—The *mixtures of amino acids* were composed of the ten acids previously found to be necessary for the growing rat.³ Practically no information was available in the literature to guide us in formulating mixtures suitable for man. After careful consideration, it was decided to employ preparations in which the component amino acids were present in approximately the ratios of the minimum levels necessary for maximum growth in the rat (23, 24). Incidentally, such ratios are not very different, in most instances, from those which actually occur in

² The authors are deeply indebted to Dr. Van Slyke for sending us detailed directions for this excellent method before the latter had appeared in print.

³ In later experiments the number of amino acids in the basal mixtures was less than ten, for reasons which will be explained at the appropriate time.

casein. A departure from the minimum levels was made in the case of two amino acids, namely tryptophan and arginine. Because of the relatively small amounts of these compounds which are required by the rat, the proportion of each was moderately increased in the human diet. Subsequent events demonstrated that this was unnecessary, as will be made clear in later papers of this series.

In preparing the mixtures, sufficient quantities of the air-dried components were carefully weighed and ground together in a ball mill for approximately 12 hours. The resulting powder was then analyzed, and an amount which would furnish exactly the desired quantity of nitrogen was administered to each subject daily. For this purpose, the acids were dissolved in distilled water, with warming if necessary. One-third of the resulting solution was consumed slowly, along with the remainder of the diet, at each of the three meals.

Many attempts were made to improve the taste of the amino acid solutions. It was finally observed that the subjects experienced the least difficulty when centrifuged lemon juice was employed as the flavoring agent. The juice was prepared from fresh lemons, filtered through cheesecloth, and centrifuged at 1200 R.P.M. for about 15 minutes. The supernatant liquid was poured off and the residue was discarded. Usually 100 ml. were taken by each subject daily. Analysis showed that the quantity specified contained approximately 45 mg. of unknown nitrogen. Many subjects found that the taste of the solutions was further improved by the addition of sucrose. Considerable freedom was permitted as to the amount of sucrose used for this purpose. However, in all cases, the diet of a given subject, once established, was kept uniform throughout the experiment except as specified below. At first, sodium bicarbonate equivalent to the hydrochlorides of the basic amino acids was introduced into the food. This resulted in alkaline urines with the concomitant precipitation of phosphates. In order to prevent this condition, the bicarbonate intake was reduced until the urines remained slightly acid to litmus. In certain of the later experiments it was omitted altogether.

The subjects received a large part of their calories in the form of *wafers*. These were composed of corn-starch, sucrose, unsalted butter fat⁴ (which had been melted and centrifuged to remove particles of protein), corn oil, a salt mixture, and a baking powder prepared in this laboratory. Sodium chloride also was always added to improve the flavor of the product, and in some instances Cellu flour was incorporated to provide a residue in the alimentary tract. Amino acid solutions tend to be laxative. This effect can be controlled by adjusting the intake of Cellu flour to the needs of

⁴ Obtained from the Department of Dairy Husbandry, University of Illinois, through the courtesy of Dr. P. H. Tracy.

the subjects. Well formed stools then result. Many subjects preferred to consume the Cellu flour suspended in water rather than as a component of the wafers.

In making the wafers, the constituents were carefully weighed, mixed with water, and worked into a dough. This was rolled into a thin sheet, cut into squares, and baked until light brown. The allotment of each

TABLE I
Composition of Baking Powder

Component	Daily intake
	<i>gm.</i>
NaHCO ₃	2.05
Ca(H ₂ PO ₄) ₂ ·H ₂ O.....	2.86
Corn-starch.....	2.69
	7.60

TABLE II
Composition of Salt Mixture

Component	Daily intake
	<i>gm.</i>
CaCO ₃	1.510
KHCO ₃	2.360
MgSO ₄ ·7H ₂ O.....	0.648
FeC ₆ H ₆ O ₇ ·6H ₂ O.....	0.175
MnSO ₄ ·4H ₂ O.....	0.00222
CuSO ₄ ·5H ₂ O.....	0.00785
KI.....	0.000442

subject for each meal was then weighed and wrapped in wax-paper until used.⁵

In Table I is presented the formula of the *baking powder* employed as a leavening agent. The make-up of the *salt mixture* is given in Table II. This mixture was a modification of one described by Phillips and Hart (25), and was compounded to conform to the principal mineral constituents of milk. A correction was made to allow for the calcium and phosphorus present in the baking powder. Furthermore, the copper con-

⁵ The ingredients of the wafers were weighed by assistants in the Division of Biochemistry, and were then made into dough and baked by students from the Department of Home Economics. We are indebted to Dr. Janice M. Smith of the latter department for her cooperation in helping us obtain the services of these young women.

tent was increased to 2 mg. of the element daily, and the zinc salt and sodium chloride were omitted. As indicated above, sodium chloride was always furnished as a separate component of the wafers.

The *vitamins* were supplied in the form of cod liver oil, and pills composed of crystalline compounds supplemented with a butyl alcohol extract of Wilson's liver powder 1:20 (*cf.* Conger and Elvehjem (26)). The daily dosages are summarized in Table III. Inasmuch as the human requirements for several vitamins have not been established accurately, some guessing was necessary in compounding the pills. It was hoped that the needs of the organism for factors which were unknown, or less well defined,

TABLE III
Vitamins and Concentrates

Component	Daily intake
	mg.
Supplied in pill form	
Thiamine hydrochloride.....	3
Riboflavin.....	3
Pyridoxine hydrochloride.....	3
Nicotinamide.....	20
Calcium <i>d</i> -pantothenate.....	7
Ascorbic acid.....	100
α -Tocopherol.....	2
2-Methyl-1,4-naphthoquinone.....	2
Butyl alcohol extract of liver concentrate*	Equivalent to 5 gm. Wilson's liver powder 1:20
Supplied in cod liver oil	I. U.
Vitamin A.....	5000
" D.....	500

* Conger and Elvehjem (26).

when this investigation was inaugurated would be met by the liver concentrate. No reason exists which would lead us to believe that this aspiration was not attained, at least for the experimental periods employed in this and other studies to be described in later papers. Consequently, the composition of the pills has not been altered despite the fact that new vitamins have been revealed during the past 7 years. Incidentally, the vitamins in the butter and lemon juice were always disregarded, since the amounts present undoubtedly varied from one lot to another. The vitamin pills were generally consumed in the course of the morning meal, and the cod liver oil during the midday meal.

The *additional calories* which were required by the subjects, over and above those derived from the amino acids and wafers, were furnished in

the form of sucrose and butter fat. As has already been pointed out, part or all of the sugar was sometimes consumed in the amino acid solutions. Other subjects preferred to eat loaf sugar at the end of the meal as a substitute for a dessert. Occasionally, a simple candy was made by concentrating an aqueous sucrose solution to crystallization, and flavoring the product with a drop of oil of peppermint. Regardless of the form, the intake of sugar was accurately controlled for each individual. The extra butter fat was invariably used as a spread for the wafers. Frequent determinations of moisture in the air-dried components of the diets enabled us to calculate precisely the amount of each constituent necessary to maintain the caloric intakes at the desired levels.

A surprising feature of experiments involving the use of diets containing mixtures of amino acids in place of proteins is the fact that the subjects require rather high intakes of energy in order to maintain nitrogen balance. This is true regardless of the nature of the mixture, and the number of amino acids of which it is composed. Evidence in support of this statement, together with a discussion of the possible reasons therefor, will be presented at a later date.⁶ At the moment, the situation is mentioned merely to account for the somewhat unusual caloric intakes employed by us throughout the course of these studies. We are convinced that this practice does not minimize the significance of the findings. Indeed, one is forced to employ high caloric rations if investigations of this general nature are to be pursued successfully in man.

Rôle of Valine and Methionine—Having described in some detail the general techniques employed in all of the experiments involving the qualitative rôle of the amino acids in human metabolism, we come now to a consideration of the specific effects upon nitrogen equilibrium exerted by the removal from the food of single components.

The first amino acids to be studied were valine and methionine. Two healthy young men, 23 and 25 years of age, served as the subjects. The composition of the amino acid mixture (Mixture A) is shown in Table IV. As will be observed, it contained four racemic acids. The proportions of three of these, namely valine, isoleucine, and threonine, were doubled over the desired levels on the assumption that the D isomers might not be utilized by man. On the other hand, the quantity of DL-methionine was not altered since both optical forms are readily utilized by the rat (28). Tentatively, it was assumed that a similar situation might exist in the human species.

Each subject consumed 50.29 gm. of the mixture daily. Of this, 36.81

⁶ A brief consideration of this problem will be found in a paper presented by the senior author before the American Institute of Nutrition in Detroit on April 21, 1949 (27).

gm. were presumed to be physiologically active on the basis of the behavior of the several components in the rat. The nitrogen content of the amino acids amounted to 6.7 gm., of which 1.2 gm. were derived from the D forms of valine, isoleucine, and threonine, and consequently may not have been utilized. Thus, the intake of nitrogen of known effectiveness was quite low.

TABLE IV
Composition of Amino Acid Mixture A

Component	Daily intake		
	Physiologically active	As used	Nitrogen content
	gm.	gm.	gm.
Valine.....	4.28	8.56*	1.02
Leucine.....	4.91	4.91	0.52
Isoleucine.....	3.08	6.16*	0.66
Methionine.....	3.68	3.68*	0.35
Threonine.....	3.08	6.16*	0.72
Phenylalanine.....	4.29	4.29	0.36
Tryptophan.....	1.85	1.85	0.25
Lysine.....	6.14		
" monohydrochloride.....		7.68	1.18
Histidine.....	2.46		
" monohydrochloride monohydrate.....		3.32	0.66
Arginine.....	3.04		
" monohydrochloride.....		3.68	0.98
	36.81	50.29	6.70†

* Racemic acids.

† Of the 6.70 gm. of nitrogen contributed by the amino acids, 1.20 gm. were derived from the D isomers of valine, isoleucine, and threonine.

In Table V are presented the composition and daily intake of the wafers (Wafers I). The unknown nitrogen present in the daily allotment amounted to 0.19 gm. The unidentified nitrogen from all sources (wafers, extra butter fat, liver concentrate, and lemon juice) amounted to 0.32 and 0.34 gm. daily for the two subjects, and thus the corresponding figures for the total nitrogen content of the rations were 7.02 and 7.04 gm. daily. These slight differences were due to the fact that the caloric intakes were not identical in the two individuals. As has already been pointed out, the caloric adjustments were usually made by varying the intakes of sugar and butterfat. Subject W. J. H., with an initial body weight of 59.6 kilos, ingested daily 100 gm. of sucrose and 80 gm. of butterfat in addition to

the quantities present in the wafers. His energy intake from all sources amounted to 2890 calories. Subject J. E. J. weighed 63.7 kilos at the start of the experiment, and received a diet which was identical with that of W. J. H. except that it contained 50 gm. of extra starch. His total energy intake was 3077 calories.

After the diets had been consumed for several days, the urine and feces were quantitatively collected and analyzed as already described. The nitrogen balance data for W. J. H. are shown graphically in Fig. 1. For the entire fore period of 8 days he manifested a slight positive balance. This is quite remarkable in view of the fact that the diet furnished only

TABLE V
*Composition and Daily Intake of Wafers I**

Component	Daily intake	Approximate calories†
	gm.	
Corn-starch.....	200.00	750
Sucrose.....	30.00	120
Butter fat (melted and centrifuged).....	67.00	623
Corn oil.....	5.00	47
Salt mixture.....	4.71	
Baking powder (starch).....	7.60	11
Sodium chloride.....	10.00	
Cellu flour‡.....	15.00	
	339.31	1551

* The daily intake of Wafers I contained 0.19 gm. of unknown nitrogen.

† After correcting for moisture.

‡ Sometimes supplied separately in water suspension and in amounts varying from 15 to 25 gm.

ten amino acids. Thus, the findings demonstrate clearly that *the ten acids previously found to be dispensable for the rat (3) and the dog (29) are also dispensable for man*. At the expiration of the fore period, valine was removed from the food, and the other amino acids were increased sufficiently to provide a constant nitrogen intake. The effects of this change were profound. On the 2nd day, the subject showed a negative nitrogen balance. On the 4th day, the nitrogen output exceeded the intake by 2.19 gm. The return of valine to the food was followed promptly by a progressive decrease in the loss of nitrogen for the next 2 days, and a strong retention of nitrogen on the 3rd and succeeding days.

After 6 days upon the complete diet, methionine was excluded from the ration for a period of 6 days. Again the subject lost more nitrogen than he consumed, although the maximum negative balance (0.51 gm.) was not

so large as that which followed valine deprivation. Immediately after the return of methionine to the diet positive balance ensued.

With J. E. J. (Fig. 2) the results were similar except that he was not quite in equilibrium during the fore period. The daily fluctuations were

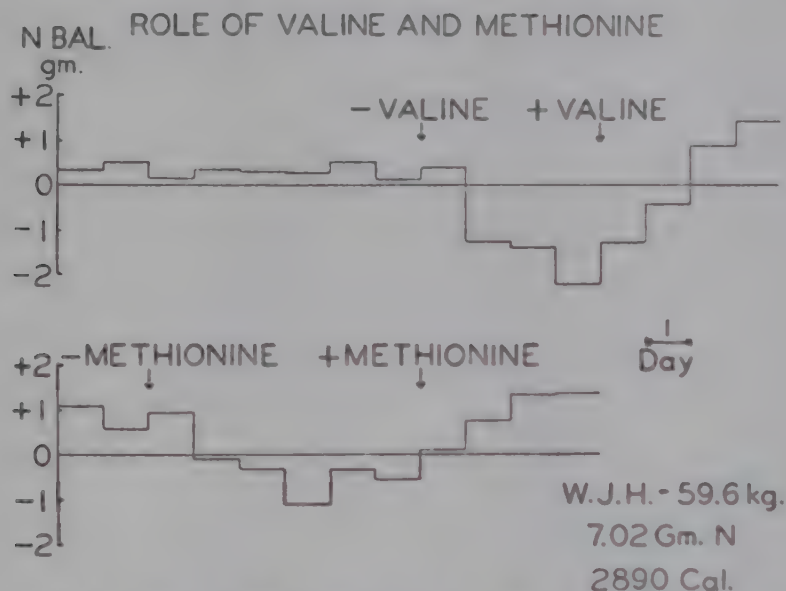


FIG. 1. The rôle of valine and methionine in the maintenance of nitrogen equilibrium in man.

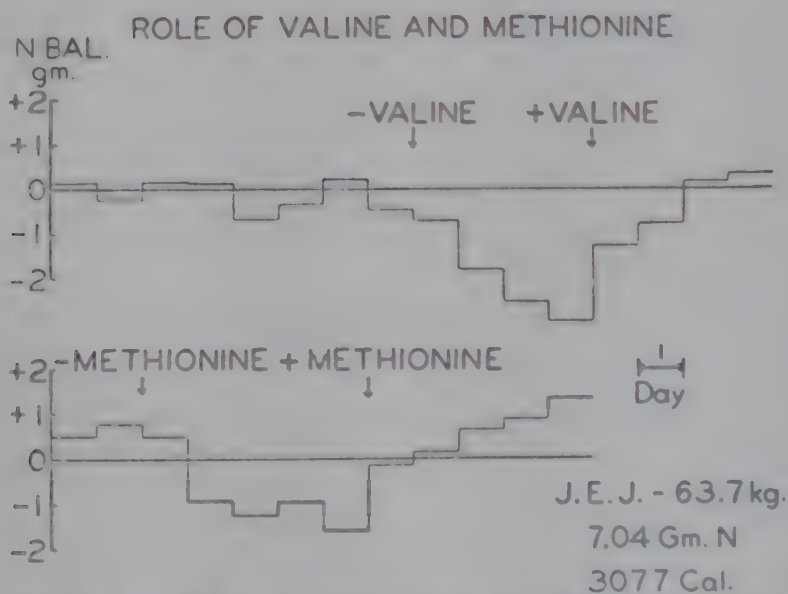


FIG. 2. The rôle of valine and methionine in the maintenance of nitrogen equilibrium in man.

rather large, but the average of the 8 day period showed a daily loss of 0.13 gm. of nitrogen. Later experiments clearly showed that the caloric intake of this subject was slightly too low for a diet containing a mixture of amino acids. However, the effects of valine and methionine deprivation are unmistakable. Following the exclusion of the former from the food, the maximum negative balance was 2.91 gm. on the 4th day. With-

out dietary methionine, the maximum nitrogen loss was 1.6 gm. on the 5th day. After both types of deficiency, the return of the missing component to the ration was followed by the establishment of positive balance.

TABLE VI

Rôle of Valine and Methionine in Nitrogen Balance

Subject, W. J. H.; daily nitrogen intake, 7.02 gm.

Day	Body weight	N output		N balance	Blood			Diet
		Urine	Feces		Non-protein N	Plasma proteins	Hemo-globin	
	kg.	gm.	gm.	gm.	mg. per cent	gm. per cent	gm. per cent	
1	59.6	5.70	1.01	+0.31				Complete; 10 amino acids
2	61.0	5.51	1.01	+0.50				
3	61.4	5.88	1.01	+0.13				
4	61.4	5.68	1.01	+0.33				
5	61.4	5.71	1.01	+0.30				
6	61.4	5.73	1.01	+0.28				
7	61.4	5.50	1.01	+0.51				
8	61.0	5.90	1.01	+0.11	30	7.4	16.0	No valine; 9 amino acids
9	61.0	5.89	0.73	+0.40				
10	61.0	7.55	0.73	-1.26				
11	60.0	7.67	0.73	-1.38				Complete; 10 amino acids
12	60.0	8.48	0.73	-2.19	49	7.3	16.8	
13	60.0	7.31	0.98	-1.27				
14	60.0	6.44	0.98	-0.40				
15	60.5	5.14	0.98	+0.90				
16	60.0	4.59	0.98	+1.45				
17	60.5	4.93	0.98	+1.11				
18	60.0	5.47	0.98	+0.57	39	7.7	16.7	No methionine; 9 amino acids
19	61.0	5.09	0.97	+0.96				
20	61.4	6.14	0.97	-0.09				
21	61.0	6.32	0.97	-0.27				
22	61.0	7.16	0.97	-1.11				
23	60.0	6.35	0.97	-0.30	47	7.4	16.4	Complete; 10 amino acids
24	60.0	6.56	0.97	-0.51				
25	60.0	5.79	1.10	+0.13				
26	60.5	5.13	1.10	+0.79				
27	60.0	4.55	1.10	+1.37				
28	60.0	4.54	1.10	+1.38	37	7.0	15.7	

These observations demonstrate that *valine* and *methionine* are essential components of the diet of man. This conclusion has been confirmed in several other subjects in connection with determinations of the minimum valine and methionine requirements.

In Table VI is shown the distribution of excretory nitrogen between the urine and feces of W. J. H. The marked rise in urinary nitrogen during both types of dietary deficiency is quite striking, as is the retention of nitrogen during the after periods, when the subject was endeavoring to compensate for the preceding nitrogen loss. Table VI also shows the results of the blood analyses made at the end of each period. The values for total plasma proteins and hemoglobin remained within the normal range throughout. On the other hand, the whole blood non-protein nitrogen increased from a normal value of 30 mg. per cent during the fore period to levels of 49 and 47 mg. per cent respectively during the valine and methionine deficiencies. Doubtless these changes merely reflect the accelerated catabolism which occurred during the dietary inadequacies. During the after periods, the values decreased, but failed to return to the normal level in

TABLE VII

Urinary Nitrogen Partition (Period Averages)

Subject, W. J. H.; initial body weight, 59.6 kilos.

Period	Total N	Urea N	Ammonia N	Creatinine N	Creatinine N	Uric acid N	α -Amino N	Rest N	Diet
days	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	
8	5.70	3.56	0.39	0.61	0.02	0.12	0.71	0.29	Complete
4	7.40	5.42	0.45	0.58	0.02	0.13	0.59	0.21	No valine
6	5.65	3.77	0.31	0.56	0.01	0.10	0.71	0.19	Complete
6	6.27	4.31	0.33	0.55	0.01	0.12	0.72	0.23	No methionine
4	5.00	2.75	0.49	0.54	0.01	0.11	0.78	0.32	Complete

the time available. The results of the erythrocyte and leucocyte counts are not presented inasmuch as they were perfectly normal throughout the course of the experiments. The corresponding data for urinary and fecal nitrogen, blood proteins, non-protein nitrogen, and cell counts obtained with J. E. J. are omitted to conserve space. In all particulars, the values were strictly analogous to those observed with W. J. H.

In Table VII are summarized data on the distribution of nitrogen between the various urinary components in subject W. J. H. Again, the findings with J. E. J. were entirely comparable, and are omitted for the sake of brevity. A like reason is responsible for the use in Table VII of period averages, although determinations of all components were made daily. As might be anticipated, the output of α -amino nitrogen was high. It should be recalled that the subjects ingested daily 1.2 gm. of α -amino nitrogen in the form of D-valine, D-isoleucine, and D-threonine. Probably, these compounds were only partially utilized; hence, their excretion may

account in large measure for the output of α -amino nitrogen. This suggestion is rendered more plausible by the fact that when valine was removed from the food, with a resulting decrease of about 0.4 gm. in the intake of nitrogen in the form of D-amino acids, both subjects showed a drop in urinary α -amino nitrogen. No such decrease occurred in either subject when DL-methionine was excluded from the ration. These facts appear to provide indirect evidence for a difference in the extent to which D-valine and D-methionine are utilized by man. Direct evidence of a similar sort will be presented at a later date.

With respect to the other urinary constituents, nothing very striking is to be observed. Indeed, the output of most of them is just as one would expect with a normal diet of comparable nitrogen content. Traces of creatine were found in the urines of both subjects almost invariably. This is contrary to our experience with normal adult males when the samples have been analyzed by the older visual colorimetric techniques. We are inclined to attribute the present findings to the superior accuracy of the photoelectric method, but realize that authorities are not in agreement regarding the presence of creatine in normal urines (*cf.* (30, 31)).

With respect to the symptoms exerted by a deficiency of valine or methionine, little of a *specific* nature can be described. Invariably, the exclusion of an essential amino acid from the food of human subjects led to a profound failure in appetite, a sensation of extreme fatigue, and a marked increase in nervous irritability. These symptoms were experienced even when the subjects were unaware that a dietary alteration had been made. Furthermore, with the return of the missing amino acid to the food, the subjective symptoms disappeared, usually within 24 hours. Like results followed both types of deficiency described in the present paper, but appeared to be somewhat more pronounced after valine deprivation than following the exclusion of methionine. This is of interest in view of the variation in the extent of negative nitrogen balance induced by the absence of the two amino acids. On the other hand, no symptoms which were *characteristic* of a specific amino acid shortage were observed in this or in any of our human experiments. Nor should one expect otherwise. All tests were of relatively short duration. They had to be, since the loss of appetite rendered it impossible for the subjects to consume the deficient diets for longer periods of time. Even so, the performance by the young men of their routine classroom and laboratory responsibilities was far from easy. Possibly, an experimental régime involving a mild deficiency could be endured for a sufficiently long interval to reveal unique physiological or psychological changes attributable to individual dietary inadequacies. However, disclosures of this character were not the primary purposes of the present investigation.

SUMMARY

The general procedures followed in investigating the amino acid requirements of human subjects, and the composition of a basal diet suitable for this purpose, have been described.

Adult male subjects receiving such a diet, in which 95 per cent of the total nitrogen is furnished by a mixture of the ten amino acids previously found to be essential for the rat, establish nitrogen equilibrium promptly, or manifest a slight positive balance, provided the caloric intake also is adequate. This demonstrates that *the ten amino acids which are dispensable for the growth of the rat are also dispensable for the maintenance of nitrogen equilibrium in human adults.*

The removal from the above ration of either valine or methionine, without altering the total nitrogen intake, induces a pronounced negative balance which is relieved by the return to the diet of the missing amino acid. This provides convincing proof that *valine and methionine are indispensable components of the diet of man.*

BIBLIOGRAPHY

1. McCoy, R. H., Meyer, C. E., and Rose, W. C., *J. Biol. Chem.*, **112**, 283 (1935-36).
2. Meyer, C. E., and Rose, W. C., *J. Biol. Chem.*, **115**, 721 (1936).
3. Rose, W. C., Oesterling, M. J., and Womack, M., *J. Biol. Chem.*, **176**, 753 (1948).
4. Borman, A., Wood, T. R., Black, H. C., Anderson, E. G., Oesterling, M. J., Womack, M., and Rose, W. C., *J. Biol. Chem.*, **166**, 585 (1946).
5. Scull, C. W., and Rose, W. C., *J. Biol. Chem.*, **89**, 109 (1930).
6. Berg, C. P., Rose, W. C., and Marvel, C. S., *J. Biol. Chem.*, **85**, 207 (1929-30).
7. Rose, W. C., and Rice, E. E., *J. Biol. Chem.*, **130**, 305 (1939).
8. Rose, W. C., and Womack, M., *J. Biol. Chem.*, **166**, 103 (1946).
9. Rose, W. C., Haines, W. J., and Johnson, J. E., *J. Biol. Chem.*, **146**, 683 (1942).
10. Rose, W. C., Haines, W. J., Johnson, J. E., and Warner, D. T., *J. Biol. Chem.*, **148**, 457 (1943).
11. Rose, W. C., *Proc. Am. Phil. Soc.*, **91**, 112 (1947).
12. Scales, F. M., and Harrison, A. P., *J. Ind. and Eng. Chem.*, **12**, 350 (1920).
13. Chibnall, A. C., Rees, M. W., and Williams, E. F., *Biochem. J.*, **37**, 354 (1943).
14. Folin, O., and Youngburg, G. E., *J. Biol. Chem.*, **38**, 111 (1919).
15. Folin, O., and Bell, R. D., *J. Biol. Chem.*, **29**, 329 (1917).
16. Folin, O., *J. Biol. Chem.*, **17**, 469 (1914).
17. Benedict, S. R., and Franke, E., *J. Biol. Chem.*, **52**, 387 (1922).
18. Christman, A. A., and Ravitch, S., *J. Biol. Chem.*, **95**, 115 (1932).
19. Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P. B., *J. Biol. Chem.*, **150**, 251 (1943).
20. Koch, F. C., and McMeekin, T. L., *J. Am. Chem. Soc.*, **46**, 2066 (1924).
21. Koch, F. C., *Practical methods in biochemistry*, Baltimore, 3rd edition, 127 (1941).
22. Sanford, A. H., Sheard, C., and Osterberg, A. E., *Am. J. Clin. Path.*, **3**, 405 (1933).
23. Rose, W. C., *Science*, **86**, 298 (1937).

24. Rose, W. C., Smith, L. C., Womack, M., and Shane, M., *J. Biol. Chem.*, **181**, 307 (1949).
25. Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, **109**, 657 (1935).
26. Conger, T. W., and Elvehjem, C. A., *J. Biol. Chem.*, **138**, 555 (1941).
27. Rose, W. C., *Federation Proc.*, **8**, 546 (1949).
28. Jackson, R. W., and Block, R. J., *J. Biol. Chem.*, **122**, 425 (1937-38).
29. Rose, W. C., and Rice, E. E., *Science*, **90**, 186 (1939).
30. Dill, D. B., and Horvath, S. M., *Am. J. Physiol.*, **133**, 520 (1941).
31. Peters, J. H., *J. Biol. Chem.*, **146**, 179 (1942).

PYRIDOXAL PHOSPHATE AND PYRIDOXAMINE PHOSPHATE AS GROWTH FACTORS FOR LACTIC ACID BACTERIA*

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During an investigation of the nutritional requirements of lactic acid bacteria two species were encountered which did not grow in a medium considered, at the time, to be complete for the growth of this group of organisms, but which grew heavily when natural materials were added. From the properties of the unidentified growth factor, we were led to examine the calcium salt of pyridoxamine phosphate for activity. This substance, in very small amount, supported growth almost as extensive as that achieved with natural materials. These observations were reported briefly at that time (1). Subsequent work has demonstrated that these organisms have another requirement which, when satisfied, corrects the variability encountered in some of the earlier experiments. The present article presents the experimental conditions under which these requirements may be demonstrated, together with information concerning the specificity of the response to them.

EXPERIMENTAL

Test Organisms—One strain each of *Lactobacillus helveticus* and of *Lactobacillus acidophilus* was first investigated.¹ *Lactobacillus delbrueckii*, ATCC 9649, has been used more recently. Stock cultures were carried in litmus milk containing 0.5 per cent glucose and 0.5 per cent Difco yeast extract. Cultures were incubated for 24 hours, or until they became acid to litmus, and were then stored in the refrigerator. Transfers were made biweekly.

Inoculum Medium—5 ml. portions of medium were used for growing the inoculum. This medium contained, per 5 ml., 2.5 ml. of basal medium (Table I), Difco proteose-peptone (25 mg.), Difco yeast extract (8 mg.), and extract of malt sprouts (4 mg.). The last was prepared by soaking ground malt sprouts in 10 volumes of water for several hours and filtering

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¹ These cultures were from the collection of Dr. W. B. Sarles, Department of Agricultural Bacteriology, University of Wisconsin.

through a pad of filter aid. The dry weight of an aliquot was determined, and the appropriate volume of solution added to the medium.

TABLE I
Basal Medium

Ingredients	Amount per 5 ml.; double strength*	Ingredients	Amount per 5 ml.; double strength*
	<i>mg.</i>		<i>mg.</i>
Glucose	100	Adenine·H ₂ SO ₄	0.1
Sodium acetate	100	Guanine·HCl	0.1
“ citrate·5½H ₂ O	100	Uracil	0.1
KH ₂ PO ₄	30	Tween 80	10
K ₂ HPO ₄	30	Oleic acid	0.1
NaCl†	51		γ
MgSO ₄ ·7H ₂ O	28	Riboflavin	4
MnSO ₄ ·H ₂ O	5.6	Ca pantothenate	4
FeSO ₄ ·7H ₂ O	1.4	Niacin	4
HCl-hydrolyzed casein‡	50	Thiamine chloride·HCl	2
Pancreatin-digested casein§	6	p-Aminobenzoic acid	2
L-Asparagine	1	Pyridoxal·HCl	2
D,L-Tryptophan	1	Pyridoxamine·2HCl	2
L-Cystine	2	Folic acid	0.1
		Biotin	0.02

* For convenience the various ingredients were made up in five stock solutions as follows: Solution 1 contained glucose, sodium acetate, sodium citrate, and inorganic salts; Solution 2, oleic acid (0.1 per cent) in 10 per cent Tween 80 solution; Solution 3, HCl-hydrolyzed casein, amino acids, purine bases, and uracil; Solution 4, pancreatin-digested casein, and Solution 5, vitamins. The first two solutions were preserved at -4°, the latter three under toluene in the refrigerator. Appropriate volumes to supply the indicated quantities per 5 ml. were mixed, heated to effect solution of any precipitated ingredients, adjusted with sodium hydroxide to pH 6.5, and diluted to volume.

† This value was determined by making a direct analysis of the medium for sodium and subtracting from the value so obtained that amount of sodium which was introduced in the form of sodium acetate and sodium citrate. It represents, principally, the salt introduced in neutralizing the HCl-hydrolyzed casein with NaOH. The concentration of this ingredient varies from one lot of medium to another and is not critical.

‡ Prepared as described previously (2).

§ Prepared as described previously (3).

Inoculum—A loop transfer was made from the stock culture to 5 ml. of inoculum medium. After incubation at 37° for 30 hours the cells were washed twice with 5 ml. of 0.9 per cent NaCl solution and diluted in a 2 cm. tube to give 90 per cent transmission of the incident light, with the Evelyn colorimeter. 1 drop of this suspension was used to inoculate each experimental tube (10 ml.).

Basal Medium and Assay Procedure—The composition of the basal medium is presented in Table I. The samples were pipetted into 16 × 180 mm. test-tubes containing 5 ml. of the double strength medium. Water was added to give a final volume of 10 ml. Each tube was well mixed to avoid local variations in the concentration of the medium during the autoclaving process. The tubes were covered with aluminum caps and autoclaved at 120° for 5 minutes. When substances were sterilized by filtration, they were added in a volume of 0.1 ml. to 9.9 ml. of the cooled, autoclaved medium, to avoid appreciable differences in concentration of the medium during autoclaving. After cooling and inoculating, tubes were incubated at 37° for 24 hours. Growth was measured turbidimetrically in 1.8 cm. tubes with the Evelyn colorimeter and the 660 m μ filter.

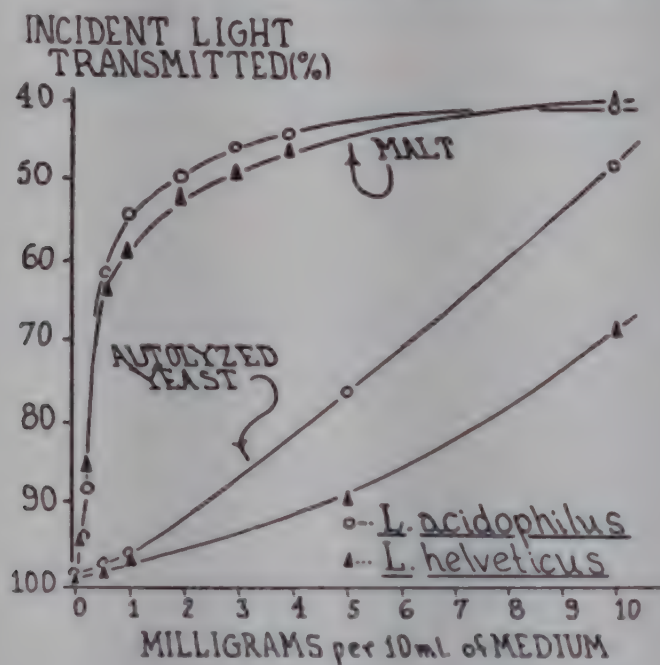


FIG. 1. Comparative response of *L. helveticus* and *L. acidophilus* to addition of natural materials to the deficient basal medium.

Results

Activity of Natural Materials—The comparative response of *L. helveticus* and of *L. acidophilus* to natural materials is shown in Fig. 1. With *L. helveticus* as the test organism and malt sprouts as the source material, the active material was found to fractionate as summarized in Table II. Since the activity of concentrates was rapidly destroyed by exposing preparations to light and since a malt preparation of high phosphatase activity rapidly lost the ability to promote growth of *L. helveticus* when incubated with water, whereas the boiled solution of the same sample showed no such loss of activity, it was thought advisable to test the phosphate esters of light-sensitive vitamins for ability to support growth of the organism.

Activity of Known Compounds—The results summarized in Table III show that pyridoxal phosphate and pyridoxamine phosphate were highly

active in replacing natural materials. A sample of pyridoxamine phosphate prepared by heating pyridoxal phosphate with excess glutamic acid (5) was 6 to 8 times more active than pyridoxal phosphate for both organisms. The activity of a sample of pyridoxamine phosphate prepared by direct phosphorylation of pyridoxamine² in the Merck laboratories checked that of the sample prepared by heating glutamic acid with pyridoxal phosphate. Since the synthetic preparations of pyridoxal phosphate and of pyridoxamine phosphate were of similar purity, as indicated by the amount of vitamin B₆ liberated following acid hydrolysis (cf. Table III), this provides direct evidence for the identity of pyridoxamine phosphate

TABLE II
Fractionation of Active Material from Malt Sprouts

Concentration procedure	Over-all yield of activity	Concentration achieved
	<i>per cent</i>	<i>units per mg. solids*</i>
Extraction of ground malt sprouts with 10 volumes water for 2 hrs.	100†	5
Adsorption on Filtrol at pH 2 to 3 (2 gm. Filtrol per gm. solids)	90 (Adsorbed)	
Elution with 30% pyridine (10 gm. solution per gm. Filtrol) at 25° for 12 hrs.	70	60
Pptn. with excess of basic lead acetate at pH 7; addition of equal volume 95% ethanol (filtrate discarded); ppt. decomposed with H ₂ S	45	120
Pptn. with 5 volumes acetone at 0° for 8 hrs. (supernatant discarded)	35	200

* Ground malt sprouts were assigned an activity of 1 unit per mg.

† By arbitrary designation.

with the product formed when pyridoxal phosphate is heated with glutamic acid, an identity previously assumed (5) on the basis of indirect evidence. The comparative response of *L. helveticus* to crude malt sprouts and to the vitamin B₆ phosphates is shown in Fig. 2. The presence of only 1 γ of pyridoxamine phosphate (which has been shown to occur naturally (5)) per gm. of malt sprouts would account quantitatively for the growth-promoting action of the latter material for *L. helveticus*; and the similar shape of the two dose-response curves provides further evidence for the identity of the naturally occurring growth factor with pyridoxamine phosphate. Pyridoxal phosphate also contributes to the activity of natural materials.

² We are indebted to Dr. Karl Folkers for this sample and for information concerning its preparation.

TABLE III

Relative Activities of Pyridoxal Phosphate and Pyridoxamine Phosphate

All the samples shown in this table, except the hydrolyzed sample, were sterilized by being autoclaved separately with water. The appropriate weight of sample in a volume of 0.1 ml. was added to the cool medium.

Addition to basal medium	Amount per 10 ml.	Per cent incident light transmitted*	
		<i>L.</i> <i>helveticus</i>	<i>L.</i> <i>acidophilus</i>
None	γ	82	94
Pyridoxal phosphate (calcium salt)†	0.005	74	
	0.01		84
	0.02	56	
	0.05	51	51
	0.1		49
Pyridoxamine phosphate‡ prepared by transamination	0.0005	78	
	0.002	70	
	0.005	49	52
	0.01	46	50
	0.05		49
Transaminated sample following hydrolysis§ Pyridoxamine phosphate (calcium salt)†	0.3	83	
	0.0005	80	
	0.001	75	
	0.004	49	
	0.005		55
	0.01		48

* Distilled water = 100.

† Gifts of unknown purity from Merck and Company. Dr. J. C. Rabinowitz made microbiological analyses of the samples by described procedures (4). The pyridoxal phosphate sample had a free vitamin B₆ activity (as pyridoxine hydrochloride) of 0.033 γ per microgram of compound. Following hydrolysis, 0.30 γ of pyridoxal hydrochloride was present per microgram of compound. Assuming the increased quantity of pyridoxal to be a measure of the "purity" of the sample, a calculated value of 71 per cent monocalcium pyridoxal phosphate is obtained. A like calculation with the pyridoxamine phosphate sample (0.01 γ of free vitamin B₆ activity per microgram of compound before, and 0.40 γ of pyridoxamine dihydrochloride per microgram of compound after hydrolysis) gives a value of 87 per cent monocalcium pyridoxamine phosphate.

‡ Pyridoxal phosphate (300 γ), glutamic acid (30 mg.), water, and NaOH to make 2 ml. of solution at pH 7.0 were heated at 120° for 1 hour (5).

§ 1 ml. of the transaminated sample (150 γ of the phosphate) was heated at 120° for 2 hours with 150 ml. of 0.055 *N* HCl (6).

Several other phosphorylated vitamins and related compounds were tested for activity. Flavin-adenine dinucleotide, cocarboxylase, and various nucleotides were inactive. Preparations of coenzyme I (40 per cent pure) and coenzyme II (20 per cent pure) showed low activity, somewhat

less than 1 per cent of that of pyridoxamine phosphate. Since these were impure isolates from natural materials, and since their activity was unaffected by autoclaving, their slight activity was assumed to be due to contamination with traces of pyridoxamine phosphate.

Rôle of Reducing Agents, Desoxyribosides, and Vitamin B₁₂ in Response to Vitamin B₆ Phosphates—The preceding results were obtained with a single lot of stock solution No. 1. When different lots of this stock solution were employed, *L. helveticus* and *L. acidophilus* responded feebly or

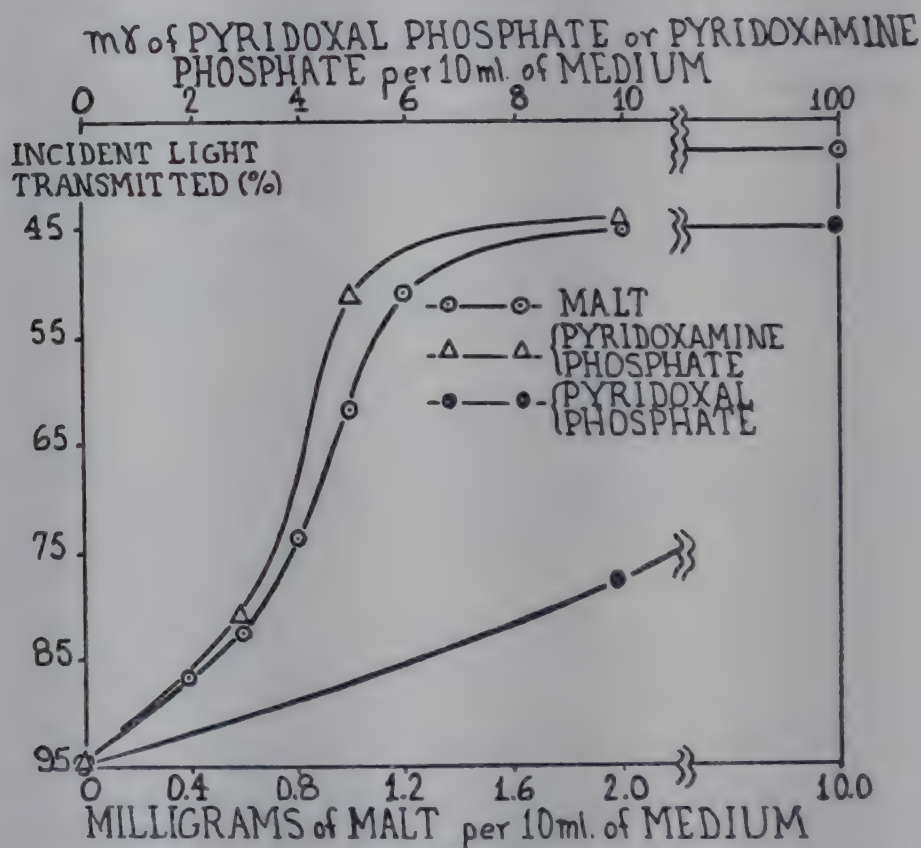


FIG. 2. Comparative activities of pyridoxamine phosphate, pyridoxal phosphate, and malt sprouts in promoting growth of *L. helveticus*.

not at all to pyridoxamine phosphate, but grew well when an extract of malt sprouts was added to the medium. An additional nutritional requirement of the test organism was thus indicated, although the variations in results with different lots of medium remained unexplained.

After many experiments, it was found that addition of various desoxyribosides, of vitamin B₁₂, or of reducing agents to the medium was essential for growth, and when any one of the additions was made, the essential nature of pyridoxamine phosphate (or pyridoxal phosphate) for growth could again be demonstrated with the two test organisms considered earlier (Tables IV and V). Growth with pyridoxamine phosphate as the sole supplement to the basal medium could be readily obtained simply by autoclaving the medium at 120° for 15 minutes (Table IV). Reducing

substances formed in this manner apparently have the same favorable effect upon growth as do added reducing agents such as ascorbic acid. It seems entirely reasonable to suppose that our early success in growing *L. helveticus* and *L. acidophilus* without added desoxyribosides, vitamin B₁₂, or reducing agents resulted from the fact that methods then in use produced the reducing environment necessary for growth under these conditions. The equivalence of reducing agents, desoxyribosides, and vitamin B₁₂ in permitting growth of these organisms is similar to that reported for several other lactic acid bacteria (9-12).

TABLE IV

Effect of Reducing Agents on Growth Response to Pyridoxamine Phosphate

The reducing agents were sterilized by filtration and added aseptically to the cool medium.

Addition to basal medium	Amount per 10 ml.	Per cent incident light transmitted		
		<i>L. helveticus</i>	<i>L. acidophilus</i>	<i>L. delbrueckii</i>
None		100	100	100
Pyridoxamine phosphate	0.01 γ	99	100	100
Ascorbic acid	3.0 mg.	92	94	96
Cysteine hydrochloride	5.0 "	93		
Glutathione*	6.0 "	80		
Pyridoxamine phosphate	0.01 γ			
+ ascorbic acid	3.0 mg.	28	25	95
+ cysteine·HCl	5.0 "	28		
+ glutathione*	6.0 "	26		
Pyridoxamine phosphate†	0.01 γ	35†		

* Obtained from the Eastman Kodak Company.

† The growth obtained when the medium was autoclaved for 15 minutes at 120°.

A survey of several organisms revealed that *L. delbrueckii* 9649 also required pyridoxamine phosphate for growth (Table V). This organism differs from the other two in that it requires thymidine for growth; neither vitamin B₁₂ nor reducing agents permitted growth in place of thymidine (*cf.* (10)).

Specificity of Requirement for Pyridoxamine Phosphate—In appropriately complete media, the vitamin B₆ requirement of some lactic acid bacteria is eliminated by addition of D-alanine to the medium (13, 14). The data of Table VI show that the requirement of *L. helveticus* and *L. delbrueckii* for phosphorylated vitamin B₆ is also eliminated by D-alanine under the conditions used. L-Alanine is inactive. These same experiments show that this response to D-alanine also occurred when the enzymatic digest of casein was omitted from the medium. In this respect, these organisms thus resemble *Streptococcus faecalis* rather than *Lactobacillus casei* (13).

TABLE V

Comparative Effects of Desoxyribosides and Vitamin B₁₂ on Growth Response to Pyridoxamine Phosphate*

Experiment No.	Addition to basal medium	Amount per 10 ml.	Per cent incident light transmitted		
			<i>L. helveticus</i>	<i>L. acidophilus</i>	<i>L. delbrueckii</i>
1	None	γ	100	100	100
	Pyridoxamine phosphate	0.01	98	100	100
	Thymidine	50	99	98	99
	Vitamin B ₁₂	0.01	98	99	100
	Pyridoxamine phosphate	0.01			
	+ thymidine	50	41	26	38
	+ vitamin B ₁₂	0.01	44	28	100
2†	Pyridoxamine phosphate	0.01	100	100	
	" "	0.01			
	+ thymidine	50	40	26	
	+ adenine desoxyriboside	50	40	28	
	+ hypoxanthine desoxyriboside	50	40	27	
	+ guanine desoxyriboside	50	42	27	
	+ cytosine desoxyriboside	50	39	24	

* We are indebted to Dr. T. G. Brady for a sample of adenine desoxyriboside, and to the P. A. Levene collection (through the courtesy of Dr. A. E. Mirsky and Dr. D. W. Woolley) for samples of guanine desoxyriboside and cytosine desoxyriboside picrate. Cytosine desoxyriboside was obtained by decomposing the picrate in the usual way. Thymidine (m.p. 185–186°, $[\alpha]_D^{25}$ (1 per cent solution in 1 N NaOH) = +31.2°) and hypoxanthine desoxyriboside (decomposition, 201–202°, $[\alpha]_D^{25}$ (1 per cent solution in water) = –20°) were isolated from sodium desoxyribonucleate by described procedures (6, 7).

† The medium employed in this experiment with *L. helveticus* was modified by substituting the amino acid mixture of Henderson and Snell (8) for the acid and enzymatic hydrolysates of casein contained in the medium of Table I.

TABLE VI

Equivalence of D-Alanine and Pyridoxamine Phosphate As Growth Factors for *L. helveticus* and *L. delbrueckii* in Complete Medium

For these tests the basal medium was modified by adding 50 γ of thymidine and omitting the pancreatin-digested casein.

Addition to basal medium	Amount per 10 ml.	Per cent incident light transmitted	
		<i>L. helveticus</i>	<i>L. delbrueckii</i>
None		100	100
Pyridoxamine phosphate	0.01 γ	35	36
L-Alanine	5 mg.	99	92
D-Alanine	5 "	45	41

DISCUSSION

The requirement of the organisms studied herein for pyridoxamine phosphate or pyridoxal phosphate, even though the basal medium used contains an excess of both pyridoxal and pyridoxamine, must mean that the specific phosphorylation mechanism involved in conversion of vitamin B₆ to its coenzyme forms is inoperative in these organisms. Only a few similar instances have previously been found. Many strains of *Hemophilus* are unable to utilize nicotinic acid or its amide for growth, but require coenzymes I or II (15). Here, however, the metabolic defect lies, not in phosphorylation, but in synthesis of the nicotinamide riboside portion of the coenzyme (16). Lankford and Skaggs (17) found that many strains of the gonococcus required cocarboxylase for growth and were unable to utilize thiamine. When such cultures were plated onto media containing suboptimal quantities of cocarboxylase, however, many of them reverted to the "normal" type which grew without cocarboxylase. No such tendency to lose the requirement for vitamin B₆ phosphates has been observed in the cultures studied here. Finally, *Acetobacter suboxydans* grows more rapidly when supplied with certain bound forms of pantothenic acid (*e.g.*, coenzyme A) than it does with free pantothenic acid (18); the rate-limiting step in synthesis of the coenzyme is not yet known.

The finding that D-alanine substitutes for vitamin B₆ phosphates in the nutrition of these organisms extends similar findings (13, 14) that D-alanine replaces vitamin B₆ under appropriate conditions for many other organisms. As emphasized elsewhere (14), this nutritional equivalence is observed only in media which contain a full complement of other compounds for syntheses of which vitamin B₆ is required, and apparently results from the fact that D-alanine is essential for growth of these organisms, but can be synthesized when the organisms are supplied adequate amounts of vitamin B₆. The same situation undoubtedly holds in this case, in which vitamin B₆ phosphate is required rather than vitamin B₆ itself.

The explanation for the equivalence of reducing agents, desoxyribosides, or vitamin B₁₂ in the nutrition of many of these organisms is not known. It has been postulated that vitamin B₁₂ is involved in synthesis of thymidine (19) or of desoxyribosides in general (10). It is possible that at or below a certain oxidation-reduction potential the organisms are able to synthesize vitamin B₁₂ (and hence desoxyribosides), just as, for example, *Neurospora sitophila* 299 synthesizes its own vitamin B₆ in a given medium only over a limited range of hydrogen ion concentrations, but requires the vitamin preformed at other pH values (20). Alternatively, the vitamin may not be required under these conditions. These questions can be answered only by further study. The specific requirement of *L. delbrueckii* for thymidine, however, illustrates the interesting differences in specificity

which occur from one organism to another. The results also emphasize the profound differences in the nutritional requirements of single organisms which may be observed, depending upon the physical and nutritional environment used.

SUMMARY

1. A strain of *Lactobacillus helveticus* and one of *Lactobacillus acidophilus* failed to grow in a medium which contained previously recognized growth factors for lactic acid bacteria, but grew well on the addition of natural materials.

2. The growth-promoting action of natural materials was duplicated by addition of pyridoxamine phosphate and any one of the following substances: (a) reducing agents, (b) thymidine or other desoxyribosides, or (c) vitamin B₁₂.

3. Pyridoxamine phosphate could be replaced by (a) pyridoxal phosphate, which was required in amounts 6 to 8 times as great, or (b) by D-alanine. Pyridoxamine and pyridoxal were inactive at the concentrations tested, concentrations far higher than are required by other organisms which require vitamin B₆, and much higher than the amounts of vitamin B₆ phosphates required.

4. The requirement for reducing agents was non-specific. It could be met by ascorbic acid, cysteine, glutathione, or simply by prolonged autoclaving of the medium.

5. A strain of *Lactobacillus delbrueckii* showed a similar requirement for pyridoxamine phosphate, pyridoxal phosphate, or D-alanine. It also showed a specific requirement for thymidine under the conditions examined.

6. The significance of these findings is discussed briefly.

BIBLIOGRAPHY

1. McNutt, W. S., and Snell, E. E., *J. Biol. Chem.*, **173**, 801 (1948).
2. Williams, W. L., Hoff-Jørgensen, E., and Snell, E. E., *J. Biol. Chem.*, **177**, 933 (1949).
3. Roberts, E. C., and Snell, E. E., *J. Biol. Chem.*, **163**, 499 (1946).
4. Rabinowitz, J. C., and Snell, E. E., *J. Biol. Chem.*, **176**, 1157 (1948).
5. Rabinowitz, J. C., and Snell, E. E., *J. Biol. Chem.*, **169**, 643 (1947).
6. Klein, W., *Z. physiol. Chem.*, **255**, 82 (1938).
7. Brady, T. G., *Biochem. J.*, **35**, 855 (1941).
8. Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, **172**, 15 (1948).
9. Shive, W., Ravel, J. M., and Eakin, R. E., *J. Am. Chem. Soc.*, **70**, 2614 (1948).
10. Kitay, E., McNutt, W. S., and Snell, E. E., *J. Biol. Chem.*, **177**, 993 (1949).
11. Hoff-Jørgensen, E., *J. Biol. Chem.*, **178**, 525 (1949).
12. Kocher, U., and Schindler, D., *Internat. Z. Vitaminforsch.*, **20**, 441 (1949).
13. Snell, E. E., *J. Biol. Chem.*, **158**, 497 (1945).

14. Holden, J. T., and Snell, E. E., *J. Biol. Chem.*, **178**, 799 (1949).
15. Lwoff, A., and Lwoff, M., *Proc. Roy. Soc. London, Series B*, **122**, 352 (1937).
16. Gingrich, W., and Schlenk, F., *J. Bact.*, **47**, 535 (1944).
17. Lankford, C. E., and Skaggs, P. K., *Arch. Biochem.*, **9**, 265 (1946).
18. Novelli, G. D., Flynn, R. M., and Lipmann, F., *J. Biol. Chem.*, **177**, 493 (1949).
19. Wright, L. D., Skeggs, H., and Huff, J. W., *J. Biol. Chem.*, **175**, 475 (1948).
20. Stokes, J. L., Foster, J. W., and Woodward, C. R., Jr., *Arch. Biochem.*, **2**, 235 (1943).

IRON AND DIHYDROXYPHENYLALANINE AS ACTIVATORS FOR LEUCYLGLYCINE DIPEPTIDASE FROM YEAST*

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Grassmann and Haag (1) separated from autolyzed yeast cells a dipeptidase preparation, which was inhibited by HCN, H₂S (2), cysteine, pyrophosphate (3), and glutathione (4). The protein precipitate, which formed on saturating solutions of this enzyme with ammonium sulfate, as well as the supernatant solution was only weakly active against leucylglycine. However, the activity of the starting material was practically recovered when both fractions were recombined (5). Similarly, treatment of dipeptidase solutions with aluminum hydroxide C γ at pH 5.0 resulted in a weakly active rest solution. The material eluted from the adsorbent at pH 7.8 also showed only slight activity, but a mixture of eluate and rest solution was considerably more active than would be expected from a mere addition of the activities of both fractions. Boiled yeast extract could be substituted for the eluate from aluminum hydroxide C γ or for the supernatant solution in the ammonium sulfate fractionation (5).

These findings were interpreted (5) as indicating that yeast dipeptidase contained an easily removable, thermostable, dialyzable coenzyme of low molecular weight.

In further work from Grassmann's laboratory, Schneider (6) described a simplified procedure for the preparation of yeast dipeptidase (free of polypeptidase) by fractional precipitation with acetone at pH 7. Treatment with acetone at pH 5 or dialysis against running water caused considerable losses in activity, which could be reversed on addition of boiled yeast extract (7).

Berger and Johnson (8) reported in 1940 that the hydrolysis of leucylglycine by crude dialyzed yeast autolysates was activated on addition of a combination of Mn or Fe ions and a reducing agent, such as cysteine, glutathione, or thioglycolic acid. Manganese ions together with cysteine formed the most effective activator. They felt (9), therefore, that the activation by boiled extract described by Grassmann *et al.* (5, 7) might be due to the presence of metal ions and reducing agents in the activating ex-

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tracts. Maschmann (10) added in 1943 the finding that L-leucylglycine dipeptidase from yeast was not activated by Mg, Mn, Co, or Zn ions, but that the slight hydrolysis of D-leucylglycine by the same enzyme preparation was markedly accelerated on addition of Mn or Fe ions together with cysteine. Maschmann (11) also contributed evidence supporting the view that there might exist an organic coenzyme for certain proteolytic enzymes. He observed that a protease from anaerobic bacteria required an activator, which was contained in the culture medium and which could not be replaced by metal ions in the presence or absence of reducing agents.

Definite proof that such a coenzyme exists and knowledge of its structure would advance significantly our understanding of proteolytic processes. The present investigation was undertaken for the purpose of establishing the chemical nature of Grassmann's activator for leucylglycine dipeptidase from yeast.

EXPERIMENTAL

Preparation of Dry Yeast—Brewers' yeast suspension (ice-cold) was filtered on Büchner funnels, washed with cold water, and sucked almost dry. The bulk of the remaining water was removed by pressing the filter cakes with a hydraulic press (350 kilos per sq. cm.), and the material was spread on sheets of filter paper and left to dry at room temperature (23–25°) for 2 days. After grinding in a ball mill for 5 hours a fine powder resulted, which was stored at 4°. Approximately 1 kilo of dried yeast powder was obtained from 2 gallons of yeast suspension.

Dipeptidase Preparation—A partially purified dipeptidase preparation was obtained as described by Schneider (6). 50 gm. of dried yeast yielded between 1.2 and 2.8 gm. of cream-colored material which showed no loss in dipeptidase activity on storage for 1 month at 4°. Depending on the particular preparation, between 0.7 and 2.0 mg. of dry powder were required per ml. of incubation mixture to hydrolyze in 1 hour 40 to 50 per cent of the L peptide, which was present in a concentration of 0.06 mM per ml. of incubation mixture. The activity of each fresh preparation was determined in a preliminary assay; thus incubation mixtures of comparable enzymic strength could be used in later experiments.

Measurement of Enzymic Activity—A stock substrate solution was prepared as follows: In a 50 ml. volumetric flask, 1.88 gm. (10 mM) of DL-leucylglycine were suspended in about 30 ml. of water and 4 ml. of 2 N NH_4OH (8 m.eq.) were added.¹ The mixture was warmed to 55° to dissolve the dipeptide more quickly. The addition of 2 ml. of 2 N H_2SO_4 and

¹ When NaOH instead of NH_4OH was used to dissolve leucylglycine, it was found in parallel experiments that the same enzyme preparation appeared up to 50 per cent less active.

water to the 50 ml. mark brought the pH of the clear solution to 7.9 to 8.0 (glass electrode).

The incubation mixtures contained 3 volumes of 0.200 M DL-leucylglycine stock solution, 1 volume of water (or of the solution to be tested for activating or inhibiting effect), and 1 volume of enzyme solution. All ingredients were warmed to 37° before mixing. A 2 ml. sample was removed immediately after addition of the enzyme and titrated with 0.05 N alcoholic KOH according to the Grassmann-Heyde procedure (12). Other 2 ml. samples were removed and titrated at intervals to follow the course of the hydrolysis. Complete hydrolysis of the L peptide contained in 2 ml. of incubation mixture (0.12 mM) was reached when 2.4 ml. of 0.05 N KOH were needed in the Grassmann-Heyde titration.

Reaction Kinetics—The enzymic hydrolysis of L-leucylglycine followed a zero order reaction course under the experimental conditions outlined above. It was found, furthermore, that the reaction velocity constant k (determined as the slope of the linear time-activity curve) was a linear function of the amount of enzyme present. The determination of k permitted, therefore, a simple evaluation of the effect of activators or inhibitors. The numerical value of k per mg. of dry powder (proteolytic coefficient) varied, of course, with the purity of the individual preparations.

Inactivation of Yeast Dipeptidase—Dialysis of 10 to 25 ml. of enzyme solution in M/600 phosphate buffer, pH 6.0, against 4 liters of the same buffer for 2 to 3 days at 4° resulted in a fairly reproducible decrease in activity (see Table I). Control solutions standing for 3 days at 4° and at pH 6.0 showed no loss in activity. The inactivation by dialysis was reversed on addition of Difco dried yeast extract or brewers' yeast extract. Schneider (13) described a method for obtaining a dry preparation by adding dropwise the viscous concentrated yeast extract to a large volume of alcohol. The resulting precipitate was filtered and dried in a vacuum desiccator (dry yeast extract). This material activated the dialyzed yeast dipeptidase, as shown in Table I. It was found, however, that the addition of saturated $\text{Ba}(\text{OH})_2$ solution to the alcohol filtrate after removal of Schneider's material produced a precipitate which was a considerably more effective activator than Schneider's fraction.

In addition to these activators from yeast, it was found that L-dihydroxyphenylalanine (dopa) exerted an activating influence on dipeptidase solutions inactivated by dialysis (see Table I).

Inhibition of Yeast Dipeptidase—The activating effect of yeast fractions, and more specifically that of dopa, seemed to support Grassmann's claim (5) of the existence of a dialyzable coenzyme for yeast dipeptidase.

In order to gain some clue as to the chemical nature of the dialyzable factor, inhibition studies were undertaken. In view of the conflicting

results of the effect of specific reducing agents on yeast dipeptidase reported by Grassmann (2-4) and by Berger and Johnson (8), the effect of cysteine and other reducing agents was investigated along with some metal reactants, such as diethyl dithiocarbamate and versene (tetrasodium salt of *N,N,N',N'*-tetracarboxymethylethylenediamine). The results of these studies are shown in Table II.

Effect of Metal Ions on Yeast Dipeptidase—The inhibition of yeast dipeptidase by metal reactants prompted an investigation of the effect of metal ions on dialyzed enzyme solutions. The results (Table III) confirmed Maschmann's observation (10) that yeast dipeptidase was not activated

TABLE I
Effect of Yeast Fractions and Dopa on Dialyzed Yeast Dipeptidase

Dialyzing time	Activator	Activator concentration	Activity
Enzyme Preparation VI, 2 mg. per ml. incubation mixture			
<i>hrs.</i>		<i>mg. per ml.</i>	<i>per cent</i>
0	None		100
45	"		52
45	Dried yeast extract according to Schneider	6.9	79
45	Ba(OH) ₂ ppt.	0.75	95
Enzyme Preparation VIII, 0.7 mg. per ml. incubation mixture			
0	None		100
0	L-Dopa	0.39	129
66	None		61
66	L-Dopa	0.39	115

Dialysis at 4° and pH 6.0; hydrolysis of leucylglycine measured after 1 hour's incubation at 37° and pH 7.9 to 8.0; activities expressed in per cent of activity found before dialysis and without addition of activator.

by Mg, Mn, Co, or Zn ions. Striking activation (110 to 130 per cent increase) was obtained, however, when Fe ions were added to dialyzed dipeptidase solutions (Table III). Considerably smaller increases in activity were observed when Fe ions were added to *undialyzed* enzyme solutions, indicating, perhaps, that part of the iron required for maximal activity had already been lost during the purification process. Seven determinations of *k* for an undialyzed dipeptidase preparation gave a value of $1.43 \pm 0.1 \times 10^{-2}$, which increased 34 per cent to $1.92 \pm 0.1 \times 10^{-2}$ when Fe ions were added (ferrous ammonium sulfate, 0.5 mM per liter).

No effect was noted on addition of Cu ions. Dopa could replace Fe ions as activator, but a concentration 20 times that of Fe was required to produce a comparable degree of activation. The Fe content of the dopa

TABLE II
Inhibition of Yeast Dipeptidase

Inhibitor	Concentration	Inhibition
	<i>mm per l.</i>	<i>per cent</i>
Sodium sulfide*	0.06	41
	0.6	55
	6.0	100
Potassium cyanide*	5.0	98
Cysteine*	4.0	96
Thioglycolic acid*	2.0	6
	20.0	97
Diethyl dithiocarbamate*	2.0	97
8-Hydroxyquinoline	0.2	25
	1.6	95
Versene	2.0	90
Hydrazine	10.0	27
Phenylhydrazine	10.0	18

Hydrolysis of leucylglycine measured after 1 hour's incubation at 37° and pH 7.9 to 8.0.

* Enzyme and inhibitor (as well as enzyme and water for the control test) were incubated for 1 hour at 37° and pH 7 prior to the addition of substrate.

TABLE III
Effect of Metal Ions on Dialyzed Yeast Dipeptidase

Dialyzing time	Material added	Hydrolysis	Activity
<i>hrs.</i>		<i>ml. 0.05N KOH</i>	<i>per cent</i>
0	None	1.21	100
66	"	0.51	42
66	FeSO ₄	1.07	88
66	CuSO ₄	0.51	42
66	ZnSO ₄	0.50	41
66	MgSO ₄	0.36	30
90	None	0.31	26
90	FeSO ₄	0.71	59
90	MnSO ₄	0.38	31
90	CoCl ₂	0.32	26
90	Dopa	0.70	58

Dialysis at 4° and pH 6.0; hydrolysis of leucylglycine measured after 1 hour's incubation at 37° and pH 7.9 to 8.0; activities expressed in per cent of activity found before dialysis and without addition of activator. The concentration of added metal compounds was 0.10 mm per liter of incubation mixture. Dopa, which was included in this table for comparison, was present in a concentration of 2.0 mm per liter of incubation mixture.

preparation used was practically nil. It was thought that both dopa and Fe might possibly be necessary to the enzyme for complete activity. How-

ever, the effect of a combination of both activators was not greater, and usually slightly less, than that of ferrous ions or dopa alone.

Contrary to results obtained by Berger and Johnson (8), who found that a combination of metal ions (Fe or Mn) and reducing agents (cysteine) acted as an activator for leucylglycine hydrolysis by crude dialyzed yeast autolysates, results from this laboratory showed inhibition of yeast dipeptidase on addition of Mn^{++} ions, cysteine, Mn^{++} ions plus cysteine, and Fe^{++} ions plus cysteine, as shown in Table IV.

TABLE IV

Comparison of Effect of Iron and Manganese Alone and in Combination with Cysteine on Dialyzed Yeast Dipeptidase

Material added	Concentration		Hydrolysis	Activity
	Metal salt	Cysteine		
	mm per l.	mm per l.	ml. 0.05 N KOH	per cent
None.....			0.87	100
$FeSO_4$	0.10		1.43	164
“ + cysteine.....	0.10	1.50	0.48	55
$MnSO_4$	1.00		0.66	76
“ + cysteine.....	1.00	3.00	0.35	40

Dialysis at 4° and pH 6.0 for 66 hours; hydrolysis of leucylglycine measured after 1 hour's incubation at 37° and pH 7.9 to 8.0; activities expressed in per cent of activity found *after* dialysis and without additions. Enzyme and additions (or enzyme and water for the control tests) were incubated for 20 minutes at pH 7.2 and 37° prior to the addition of substrate.

Maschmann's claim (10) of an activation of D-leucylglycine hydrolysis on addition of Fe plus cysteine was confirmed in the following manner. Enzymic hydrolysis of DL-leucylglycine in presence of Fe^{++} ions was carried out for 2 to 3 hours until constant titers, representing complete hydrolysis of the L component (checked polarimetrically), was obtained. Fresh enzyme and cysteine (8 mm per liter) were then added, and an increase to 40 per cent hydrolysis of the D component was observed after an incubation period of 18 hours. Without addition of cysteine less than 5 per cent additional hydrolysis took place during the same period of time.

DISCUSSION

The finding that the inactivation of yeast dipeptidase by dialysis was reversed on addition of ferrous ions makes it unnecessary to assume the existence of an easily dialyzable, organic prosthetic group for this enzyme.

The ability of dopa to act as an alternative activator is not easy to under-

stand. When ferrous ions were added to a mixture of dopa and substrate at pH 8, it was observed that a stable, deep purple color appeared, indicating complex formation. Mixing 10^{-2} M dopa solution with 10^{-4} M ferrous ammonium sulfate (pH 5.7) led to a blue color, which changed to purple on addition of base. No colored complex was formed, however, when ferrous ions were added to leucylglycine at pH 8. Smith (14) reported that Co^{++} ions, which activated glycylglycine dipeptidase, developed a pink color on incubation with the substrate. He suggested that the function of heavy metals in peptidase action is to act as a bridge in forming the enzyme-substrate compound. The relations described in this report are somewhat more involved. Fe^{++} ions did not form a colored complex with the substrate but did so with dopa. Assuming that this complex is not an inactive by-product but participates in the enzymic process, one may speculate as to its mode of action. It is conceivable that dopa facilitates the utilization of iron by the enzyme, as long as only limited amounts of ferrous ions are present, by removing iron from inert parts of the enzyme surface and donating it to the active center. This effect would disappear when an excess of Fe^{++} ions is added, conditions under which dopa shows no activating influence. That dopa might act as activator by removing, through complex formation, inhibiting metal ions from the active center of the enzyme seems less likely. The postulation of such a mechanism requires the assumption that dopa has a higher affinity for inhibiting metal ions than for iron. However, here again, an excess of ferrous ions could act similarly by displacing other inhibiting metal ions.

Our observations on the effect of dopa and iron on yeast dipeptidase recall some earlier findings of Abderhalden and von Ehrenwall (15). These authors reported that the addition of dopa (and other compounds) to trypsin solutions elicited the appearance of "ereptic" activity; *i.e.*, hydrolysis of leucylglycine. Boiled "erepsin" could be substituted for dopa to produce the same effect. When heat-inactivated "erepsin" was treated with H_2S prior to its addition to trypsin, it showed no activating ability. This inhibiting effect of H_2S suggests that the boiled "erepsin" solutions acted by virtue of their metal content. Whatever the final explanation of these phenomena will be, it is interesting that Abderhalden's studies show, similar to the work reported here, an activation of a peptidase by either dopa or metal ions.

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SUMMARY

1. The enzymic hydrolysis of leucylglycine by partially purified yeast dipeptidase was found to follow a zero order reaction course. The reaction velocity constant was a linear function of the amount of enzyme present.

2. It was confirmed that the partial inactivation of this enzyme by dialysis may be reversed on addition of various yeast extracts. A thermostable material from yeast, soluble in 95 per cent alcohol and precipitated by $\text{Ba}(\text{OH})_2$ solution, was found to be a more effective activator than the yeast fraction described by Schneider.

3. The inactivation of yeast dipeptidase by dialysis was markedly reversed on addition of Fe^{++} ions or of dihydroxyphenylalanine. The activating effects of ferrous ions and of dopa were not additive. The experiments described in this report make it unnecessary to assume the existence of a dialyzable organic coenzyme for yeast dipeptidase suggested by Grassmann and coworkers.

4. The hydrolysis of leucylglycine by yeast dipeptidase was inhibited by Na_2S , KCN, cysteine, hydrazine, phenylhydrazine, thioglycolic acid, hydroxyquinoline, diethyl dithiocarbamate, versene, and Mg^{++} ions. The hydrolysis of L-leucylglycine was inhibited and that of D-leucylglycine was activated by a combination of Fe^{++} ions and cysteine.

BIBLIOGRAPHY

1. Grassmann, W., and Haag, W., *Z. physiol. Chem.*, **167**, 188 (1927-28). Grassmann, W., *Z. physiol. Chem.*, **167**, 202 (1927-28).
2. Grassmann, W., and Dyckerhoff, H., *Z. physiol. Chem.*, **179**, 41 (1928).
3. Grassmann, W., Dyckerhoff, H., and von Schoenebeck, O., *Z. physiol. Chem.*, **186**, 183 (1930).
4. Grassmann, W., Dyckerhoff, H., and Eibeler, H., *Z. physiol. Chem.*, **189**, 112 (1930).
5. Grassmann, W., Volmer, W., and Windbichler, V., *Biochem. Z.*, **298**, 8 (1938).
6. Schneider, F., *Biochem. Z.*, **307**, 427 (1940-41).
7. Schneider, F., *Biochem. Z.*, **308**, 399 (1941).
8. Berger, J., and Johnson, M. J., *J. Biol. Chem.*, **133**, 639 (1940).
9. Berger, J., and Johnson, M. J., *J. Biol. Chem.*, **130**, 641 (1939).
10. Maschmann, E., *Naturwissenschaften*, **31**, 136 (1943).
11. Maschmann, E., *Naturwissenschaften*, **26**, 139 (1938).
12. Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, **183**, 32 (1929).
13. Schneider, F., *Biochem. Z.*, **307**, 414 (1940).
14. Smith, E. L., *J. Biol. Chem.*, **173**, 571 (1948).
15. Abderhalden, E., and von Ehrenwall, E., *Fermentforschung*, **13**, 262 (1932); **14**, 118 (1933).

THE EFFECT OF TEMPERATURE ON THE ESTERASE ACTIVITY OF CHYMOTRYPSIN

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In recent reports (1, 2) it was shown that a "secondary" peptide group in specific peptide or ester substrates for chymotrypsin was essential for the enzyme to reveal its full catalytic effect. Comparison of the α -substituted, benzamido-, hydroxyl-, and chloro-, methyl hydrocinnamates with the parent ester demonstrated that the secondary peptide bond contributed by the benzamido radical greatly enhanced the enzyme-substrate affinity as well as the specific rate of activation and that the hydroxyl group in the *l* position was capable, to a lesser extent, of exerting the same effect (1). The specific activation rates of the *d*-hydroxyl and *l*- or *d*-chloro compounds were lower, and of comparable magnitudes, whereas the unsubstituted ester, methyl hydrocinnamate, was the least active substrate of the whole series.

In order to analyze further the contribution of these α -substituents to the mechanism of chymotryptic hydrolysis, the effect of temperature on the kinetic constants,¹ K_m and k_3 (3, 4), was investigated and analyzed in terms of the changes in heat, free energy, and entropy of activation, respectively (4). The members of the substrate series were benzoyl-L-phenylalanine methyl ester, methyl *l*- β -phenyllactate, methyl *d*- β -phenyllactate, methyl *dl*- α -chloro- β -phenylpropionate, and methyl hydrocinnamate. These will be referred to herein in terms of the α -substituent as benzamido, *l*- or *d*-hydroxyl, chloro, and unsubstituted ester, respectively. For purposes of comparison, analogous data for an acetamido ester, *i.e.* acetyl-L-tyrosine ethyl ester, have also been obtained.

EXPERIMENTAL

Enzyme and substrate preparations were the same as those previously described (1). Acetyl-L-tyrosine ethyl ester was prepared by Dr. Seymour Kaufman (4). All enzymatic experiments were carried out in 0.01 M phosphate buffer, pH 7.80, containing 20 or 30 volumes per cent of methanol. Rates of ester hydrolysis were determined by potentiometric

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¹ The symbol k_3 denotes the specific rate of activation and is used herein in place of k' given in preceding publications on this subject (3-5).

titrations of the liberated carboxyl groups (6), in a total volume of 10 cc. In order to minimize the heat of dilution on the addition of the enzyme solution to the methanol-water mixture, the volume of the enzyme solution was restricted to about 0.5 cc. The temperature was varied within the range of about 10–30°.

TABLE I
Dependence of Specific Rate Constant of Ester Substrates of Chymotrypsin on Temperature

Substrate	Solvent, MeOH	Temper- ature	k_1	a
	<i>per cent</i>	°C.		$10^{-3} M$
Benzoyl-L-phenylalanine methyl ester	30	10	0.172	1.80 – 4.55 (6)
		15	0.222	2.57 – 5.45 (4)
		20	0.323	1.85 – 6.60 (5)
		25	0.543	1.89 – 5.61 (4)
		25	0.518	2.54 – 6.76 (4)
		30	0.671	2.27 – 7.02 (5)
Methyl <i>l</i> - β -phenyllactate	20	10	0.00538	5.00 – 15.00 (4)
		17	0.00894	5.00 – 15.00 (4)
		25	0.0144	5.00 – 15.00 (4)
		32	0.0220	5.00 – 15.00 (4)
Methyl <i>d</i> - β -phenyllactate	20	10	0.000407	5.00 – 15.00 (4)
		17	0.000692	5.00 – 15.00 (4)
		25	0.00144	5.00 – 15.00 (4)
		32	0.00267	5.00 – 15.00 (4)
Methyl <i>dl</i> - α -chloro- β -phenylpropionate	20	9	0.000297	0.755– 2.04 (3)
		15	0.000569	1.110– 2.545 (3)
		20	0.000840	1.010– 2.970 (4)
		25	0.00140	1.030– 2.925 (5)
Methyl hydrocinnamate	20	15	0.000107	2.74 – 8.84 (4)
		20	0.000160	2.65 – 9.27 (4)
		25	0.000268	3.05 – 9.65 (5)
Acetyl-L-tyrosine ethyl ester	30	10	0.771	3.43 – 11.50 (5)
		17	0.930	5.14 – 12.50 (5)
		25	1.97	3.86 – 15.10 (9)

a = initial substrate concentration. The numbers in parentheses in the last column refer to the number of experiments on which the calculation of k_1 , the specific rate of activation, is based.

Results

The initial velocity, v , was obtained from the initial, linear, portion of the curves obtained when the concentration of the liberated acid was plotted against time (7). For rapidly hydrolyzed substrates, the initial substrate concentration, a , was determined from the titration values after hydrolysis was allowed to proceed to completion. In the case of substrates which

were hydrolyzed at a relatively slow rate, the initial substrate concentration was taken as the analytical concentration, since the amount of hydrolysis occurring during the initial pH adjustment was negligibly small.

TABLE II
Thermodynamic Data for Hydrolysis of Ester Substrates by Chymotrypsin*

Substrate	α-Substituent	Meth- anol	k_3	$k_3^0†$	ΔE	ΔH°	ΔS°	ΔF°
		per cent	10 ⁻³	sec. ⁻¹	calories per mole	calories per mole	E.U.	calories per mole
Benzoyl-L-phenyl- alanine methyl ester	Benza- mido	30	53.0	51.0	12,500	11,900	-11.0	15,200
Methyl l-β-phenyl- lactate	Hydroxyl	20	1.43	1.38	11,000	10,400	-23.4	17,400
Methyl d-β-phenyl- lactate	"	20	0.144	0.139	15,100	14,500	-14.2	18,700
Methyl dl-α-chloro- β-phenylpropio- nate	Chloro	20	0.140	0.135	15,400	14,800	-13.2	18,700
Methyl hydrocin- namate	None	20	0.027	0.026	16,800	16,200	-11.8	19,700
Acetyl-L-tyrosine ethyl ester	Aceta- mido	30	200.0	193.0	11,500	10,900	-10.7	14,100

* The values for k_3 , k_3^0 , and ΔF^* refer to $T = 25^\circ$.

† $k_3^0 = k_3 \frac{36,000}{60 \times 6.25}$ (4).

TABLE III
Temperature Dependence of Michaelis Constant, K_m , for Chymotryptic Hydrolysis of Specific Esters

Substrate	Tempera- ture range	ΔE
	°C.	calories per mole
Methyl l-β-phenyllactate.....	10-32	6,300
" d-β-phenyllactate.....	10-32	11,100
" dl-α-chloro-β-phenylpropionate	9-25	7,600
" hydrocinnamate.....	15-25	4,400

ΔE is the critical temperature increment, calculated from $(\log K_{m2})/(\log K_{m1}) = (\Delta E/2.3R) (1/T_1 - 1/T_2)$.

The experimental data are given in Table I in which k_3 is the specific rate of activation¹ in moles per liter of substrate hydrolyzed per mg. of enzyme N in the enzyme-substrate complex (ES) per cc. (1). The last column

of Table I gives the range of initial substrate concentration which was investigated at each temperature.²

When $\log k_3$ was plotted against the reciprocal absolute temperature, the experimental points followed closely a straight line whose slope was determined by the method of least squares. The resulting value for ΔE , the Arrhenius activation energy, was interpreted in terms of the heat, free energy, and entropy of activation per unit concentration of (ES) , the Michaelis enzyme-substrate complex (4). In the calculation of ΔF^* , the free energy of activation, the molecular weight of chymotrypsin was assumed to be 36,000, although its real value, in such low concentrations in which the enzyme was present in the systems, is probably only one-half as great (8). The results³ of these calculations are given in Table II.

Interpretation of the kinetic data in terms of the two-step reaction mechanism (3, 4) yields, in addition to the specific rate of activation k_3 , also K_m , the Michaelis constant, which is likewise temperature-dependent. Since the thermodynamic significance of this constant is a matter of conjecture (4, 9), the measured effect of temperature on K_m has been expressed in Table III merely in terms of the Arrhenius activation energy, ΔE . Calculation of ΔE was restricted to those substrates for which at least three experimental points yielded a linear relation between $\log K_m$ and reciprocal absolute temperature.

DISCUSSION

Dependence of k_3 on Temperature

The energetic constants calculated from the temperature dependence of k_3 are independent of those occurring in the formation of the enzyme-substrate complex, (ES) , from the free reactants, E and S , and are solely characteristic of the activation step, $(ES) \rightarrow (ES)^*$ (4). The data given in Table II permit an analysis of the influence of the nature of the α -

² For some of these substrates, notably methyl *d*- β -phenyllactate and methyl *dl*- α -chloro- β -phenylpropionate, the kinetic constants at 25°, given in Table I, differ somewhat from those previously reported (1). As the present data are based on more extensive measurements, they are considered to be more reliable than those previously given.

³ The values for ΔH^* and ΔS^* previously reported for the hydrolysis of benzoyl-L-tyrosine ethyl ester (4) are significantly higher than those found herein for the two substrates containing a secondary peptide bond (benzoyl-L-phenylalanine methyl ester and acetyl-L-tyrosine ethyl ester). Since the latter are consistent with each other and also consistent in relation to those found for the remaining substrates of this series, the previously reported values for benzoyl-L-tyrosine ethyl ester appear to be subject to correction. The higher solubility of the present substrates containing a secondary peptide bond have admitted of a wider variation in initial substrate concentration and thus strengthened the reliability of the present data.

substituent of the parent ester, methyl hydrocinnamate, on the energetics of enzymatic activation.⁴ The effect of the substituent is 2-fold: (1) it influences the stability of the substrate *per se*, and (2) it contributes to the catalytic effect of the enzyme through combination.

The introduction of an electronegative group increases the susceptibility of esters to alkali hydrolysis by a positive inductive effect. This is evidenced by an increase in the rate and a decrease in the free energy and heat of activation (10). A similar effect can be noted in the present analyses, by comparison of methyl hydrocinnamate with the *dl*-chloro and the *d*-hydroxyl compounds, respectively. As was pointed out previously (1), the effect of these substituents cannot be related to the effect on the interaction with the enzyme, but rather is related to the influence of the substituents themselves on the stability of the substrate. The influence of the substituents containing a secondary peptide bond (the benz-amido and acetamido esters) on the ability of the substrate to combine with the enzyme is demonstrated by the higher rate and lower heat of activation of these substrates. Although the secondary peptide group is of weaker electronegativity than the chloro and the hydroxyl groups, it presumably combines with the enzyme surface by hydrogen bond formation (2).

The heat of activation for the hydrolysis of the *l*-hydroxyl compound is significantly lower than that of the *d* isomer, and somewhat lower than that of the ester substrates which contain the secondary peptide bond. Presumably, this is the result of the dual rôle of the *l*-hydroxyl group as an electronegative substituent and as a point of attachment to the enzyme.

With one exception, the free energies of activation follow the same substrate sequence as the heats of activation. This result is due to the fact that, with the same exception, the entropies of activation are nearly constant, *i.e.* approximately -11 to -14 E.U., a range which compares favorably with the data by Butler (11, 12) which are based on a less detailed analysis of the kinetic process (4). As the estimated accuracy in the determination of ΔH^* is about 1000 calories per mole, and, therefore, that of ΔS^* approximately 3.5 E.U., this variation is not significant. From the relative constancy of ΔS^* it can be concluded that the entropy of activation is unrelated to the presence or absence of the secondary peptide bond. From this finding it may be surmised that combination through the secondary peptide bond has occurred during the formation of the enzyme-substrate complex, (*ES*), rather than as part of the activation process, thereby absorbing part of the entropy change otherwise required for the activation process.

⁴ The influence of the higher methanol concentration, used in experiments with the benzamido and acetamido esters on the energetics of the kinetic process, has, as a first approximation, been neglected in the interpretation of the data.

In view of the relatively constant entropies of activation observed for all other substrates, the large decrease of -23 E.U. accompanying the activation of the *l*-hydroxyl compound requires consideration. This large, negative entropy change is responsible for the lower rate and higher free energy of activation of this ester substrate as compared to those containing the secondary peptide bond. While an interpretation of this effect must, necessarily, be conjectural, it is evident that the effect must be related to the ability of the *l*-hydroxyl group to combine with the enzyme, since the energetics of activation of the *d* isomer can be satisfactorily accounted for by the electronegative properties of this constituent. It may be tentatively proposed that combination of the *l*-hydroxyl group with the enzyme forms part of the activation process, the formation of the postulated single hydrogen bond (1) causing an additional decrease in ΔS^* . Alternatively, the large entropy decrease may be related to differences in the degree of binding of solvent molecules during the activation process (9, 13, 14).⁵ While this interpretation is necessarily restricted to qualitative terms, it may be significant that the combination of the *l*-hydroxyl group with a hydrogen bond-accepting (carbonyl) group on the enzyme surface (2) could permit the binding of water molecules, through the pair of unshared electrons of the hydroxyl oxygen, with the adjacent hydrogen bond-donating amido group of an enzyme-peptide group.

Dependence of K_m on Temperature

Interpretation of the observed variations of K_m with temperature requires consideration of the physical meaning of this constant. It has been pointed out on several occasions (1-5), and specifically for the enzymatic systems under discussion herein (1), that K_m has no direct relation to the enzyme-substrate affinity. While the relation

$$K_m = \frac{(E)(S)}{(ES)} = \frac{k_2 + k_3}{k_1} \quad (1)$$

bears formal resemblance to an equilibrium expression, it actually represents the condition for the steady state in two consecutive reactions, the first one of which is reversible (15, 16). Accordingly, since $K_m = k_2/k_1 + k_3/k_1$, a plot of $\log K_m$ against the reciprocal temperature yields a composite function which contains implicitly both a classical heat of reaction, ΔH , and a heat of activation, ΔE . There is no obvious physical model for such a function. In the light of these considerations, calculations of classical thermodynamic constants from the temperature dependence of

⁵ We are indebted to Dr. Irving M. Klotz for valuable suggestions in a discussion of this problem.

K_m , as attempted in a recent paper (4) and elsewhere,⁶ can no longer be maintained, unless it can be shown that $k_3 \ll k_2$.

In the limiting case of $k_2 \ll k_3$, equation (1) reduces to $K_m = k_3/k_1$; *i.e.*, the Michaelis constant becomes merely a ratio of the reaction constants of two consecutive reactions. This condition appears to be approximated by the system chymotrypsin-acetyl-L-tyrosinamide (5). Under such conditions, a plot of $\log K_m$ against reciprocal temperature yields the quantity ΔE , which is the difference between two heats of activation,

$$\Delta E = \Delta H^* - \Delta H^*_1 \quad (2)$$

where ΔH^* is, as before, the heat of activation for the step $(ES) \rightarrow (ES)^*$, whereas ΔH^*_1 is the activation energy for the formation of the transition complex in the reaction $E + S \rightarrow (ES)$. Since there is no evidence that for systems listed in Table III the conditions of $k_2 \ll k_3$, or $k_2 \gg k_3$, actually apply, the significance of the ΔE values listed in Table III remains indeterminate.

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SUMMARY

A study has been made of the effect of temperature on the kinetics of the chymotryptic hydrolysis of derivatives of the specific substrate methyl hydrocinnamate, which differ from each other in the nature of the α -substituents. These derivatives were benzoyl-L-phenylalanine methyl ester, and the *l*- and *d*-hydroxyl- and the *dl*-chloro-substituted compounds. In addition, the hydrolysis of the acetamido substrate, acetyl-L-tyrosine ethyl ester, was similarly investigated.

From the temperature dependence of k_3 , the specific rate of activation and the heat, free energy, and entropy of activation, respectively, have been determined. These, in turn, have been correlated with the structural characteristics of the substrates as they relate to (1) the influence of the electronegative properties of the α -substituents on the stability of the substrate, and (2) the influence of the substituents on the ability of the substrate to combine with the enzyme.

It has been shown that although the temperature dependence of the Michaelis constant, K_m , can be formally expressed by the van't Hoff equation, such data are generally devoid of physical meaning. However, under special, limiting conditions, the energetics for the formation of the transition complex in the formation of the Michaelis complex from free enzyme and substrate can be calculated from the temperature dependence of K_m .

⁶ For literature references, see Stearn (9) and Sizer (17).

BIBLIOGRAPHY

1. Snoke, J. E., and Neurath, H., *Arch. Biochem.*, **21**, 351 (1949).
2. Kaufman, S., and Neurath, H., *Arch. Biochem.*, **21**, 437 (1949).
3. Elkins-Kaufman, E., and Neurath, H., *J. Biol. Chem.*, **175**, 893 (1948).
4. Kaufman, S., Neurath, H., and Schwert, G. W., *J. Biol. Chem.*, **177**, 793 (1949).
5. Kaufman, S., and Neurath, H., *J. Biol. Chem.*, **180**, 181 (1949).
6. Schwert, G. W., Neurath, H., Kaufman, S., and Snoke, J. E., *J. Biol. Chem.*, **172**, 221 (1949).
7. Snoke, J. E., and Neurath, H., *J. Biol. Chem.*, **181**, 789 (1949).
8. Schwert, G. W., *J. Biol. Chem.*, **179**, 655 (1949).
9. Stearn, A. E., *Advances in Enzymol.*, **9**, 25 (1949).
10. Hammett, L. P., *Physical organic chemistry*, New York and London (1940).
11. Butler, J. A. V., *J. Am. Chem. Soc.*, **63**, 2971 (1941).
12. Northrop, J. H., Kunitz, M., and Herriott, R. M., *Crystalline enzymes*, New York, 2nd edition, 147 (1948).
13. Magee, J. L., Taikei, R., and Eyring, H., *J. Chem. Phys.*, **9**, 419 (1941).
14. Klotz, I. M., and Urquhart, J. M., *J. Am. Chem. Soc.*, **71**, 847 (1949).
15. Haldane, J. B. S., *Enzymes, Monographs on biochemistry*, London and New York (1930).
16. Chance, B., *J. Biol. Chem.*, **151**, 553 (1943).
17. Sizer, I. W., *Advances in Enzymol.*, **3**, 35 (1943).

ON THE PROSTHETIC GROUP OF THE D-ASPARTIC OXIDASE

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The present communication deals with the method of resolving the D-aspartic oxidase of animal tissues and with the restoration of the activity of the apooxidase by addition of flavin-adenine dinucleotide (FAD).

Results

Method of Splitting D-Aspartic Oxidase—The D-aspartic oxidase has been prepared (1) by homogenizing rabbit liver or kidney, removing the insoluble residue by centrifugation, and treating the clarified supernatant fluid with acetone. The aqueous extract of this acetone powder contains an active D-aspartic oxidase which does not require any addition of coenzyme. When the enzyme has been precipitated with ammonium sulfate at one-third saturation and the concentrated solution of the precipitate dialyzed free of salt, the activity of the oxidase is greatly reduced but can be restored by addition of FAD, as shown in Table I. The splitting may be the consequence either of the exposure to ammonium sulfate or of autolytic processes which are accelerated by concentrating both the oxidase and some enzymatic lytic factor. In the light of our previous experience with the glycine oxidase and the D-amino acid oxidase of Krebs (2) the latter explanation seems more probable. The relation between rate of oxidation and concentration of FAD is shown in Fig. 1. The saturation level of FAD is about 10 γ per cc., whereas the half saturation level is about 2.5 γ per cc.

The sample of FAD used in these experiments was prepared from yeast by the method of Warburg and Christian (3). To eliminate the possibility that the effect was due not to FAD but to some impurity in the yeast preparation a solution of FAD was obtained by heat coagulation of purified diaphorase prepared from pig heart muscle. The content of flavin dinucleotide in the protein-free extract was determined from the absorption band at 450 $m\mu$ and the coenzyme activity per microgram of FAD of this preparation was compared with activity per microgram of FAD of the standard yeast preparation. As shown in Table II, FAD prepared by two quite different methods and from two different sources

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exhibits essentially the same activity, and consequently FAD must be presumed to be the active component. Flavin monophosphate has been tested and found to show no activity as coenzyme.

TABLE I
Preparation of D-Aspartic Apooxidase

Additions		Oxygen uptake per 25 min.
		μ l.
Enzyme.....		0
“ + DL-aspartate (100 μ M).....		14
“ + 36 γ FAD.....		1
“ + DL-aspartate + FAD.....		195

The additions were 0.25 cc. of rabbit kidney enzyme, 1.0 cc. of 0.1 M phosphate buffer of pH 7.5, 0.2 cc. of 10 per cent ethyl alcohol. Total volume 3.0 cc. Air in gas space.

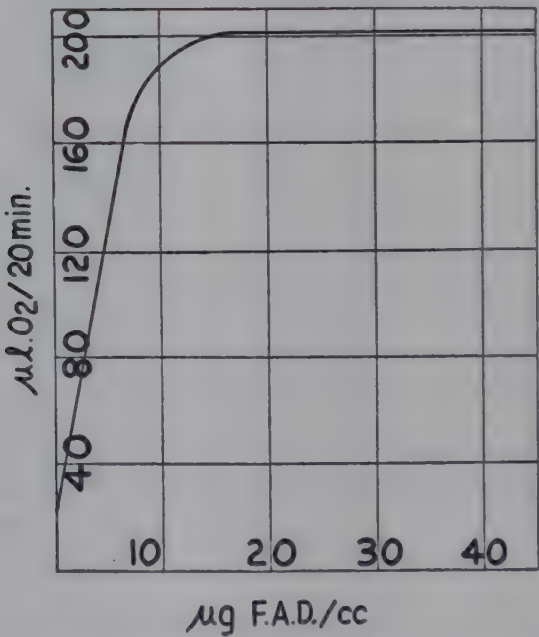


FIG. 1. Activity of D-aspartic apooxidase as a function of the concentration of FAD. Each manometer contained 1.0 cc. of split kidney enzyme, 1.0 cc. of 0.1 M phosphate buffer of pH 7.5, 0.2 cc. of 4 per cent ethyl alcohol, and 100 μ M of DL-aspartate in a final volume of 3.0 cc.

D-Glutamic Activity—The standard preparation of the D-aspartic oxidase from rabbit liver or kidney (aqueous extract of acetone powder) catalyzes the oxidation of D-glutamic acid, although at about 30 per cent of the rate with D-aspartic acid. It was of interest to determine whether a preparation of the D-aspartic apooxidase supplemented with FAD would also be active towards D-glutamic acid. Table III shows that the ratio of aspartic to glutamic activity has increased from its original value of 3.5

for the unsplit oxidase to values of 6 and 12 at pH 7.5 and 9.0 respectively for the split oxidase. This observation is not consistent with the view that the same enzyme catalyzes the oxidation of both substrates. There is additional evidence which suggests that two separate oxidases are implicated. When the D-aspartic oxidase is prepared from pig kidney, the

TABLE II
Coenzyme Activity of FAD Prepared from Yeast and Pig Heart Muscle

Source of FAD (5.5 γ)	Oxygen uptake of split oxidase, O ₂ per 20 min.
	μ l.
Yeast.....	64
Heart muscle.....	60

The additions were 1.0 cc. of rabbit kidney enzyme, 1.0 cc. of 0.1 M phosphate buffer of pH 7.5, 0.5 cc. of 0.2 M DL-aspartate, and 0.2 cc. of 10 per cent ethyl alcohol. Final volume 3.7 cc. The values in the table have been corrected for the small blank without FAD but with substrate.

TABLE III
Oxidation of D-Glutamate by Preparations of D-Aspartic Apooxidase

Substrate	pH of reaction	Oxygen uptake	
		10 min.	20 min.
		μ l.	μ l.
None.....	7.5	0	0
DL-Aspartate (100 μ M).....	7.5	139	231
DL-Glutamate (100 μ M).....	7.5	27	45
None.....	9.0	0	0
DL-Aspartate (100 μ M).....	9.0	118	262
DL-Glutamate (100 μ M).....	9.0	12	21

The additions were 0.5 cc. of the split rabbit kidney enzyme, 1.0 cc. of 0.1 M phosphate buffer of pH 7.5 or 1.0 cc. of 0.2 M aminopropanediol buffer of pH 9.0, 0.5 cc. of FAD (2.3 γ per cc.), 0.2 cc. of 10 per cent ethyl alcohol. Final volume 3.0 cc.

activity of the enzyme preparation towards D-glutamic acid is extremely low and is for all practical purposes negligible. Either the specificity of the D-aspartic oxidase is different, depending upon the source of the enzyme, or two separate oxidases are concerned, both of which are flavo-proteins present in variable amounts in the organs of different species.

D-Amino Acid Oxidase in Relation to D-Aspartic Oxidase—In rabbit liver and kidney the D-aspartic oxidase is present in relatively high concentration compared to the D-amino acid oxidase of Krebs (*cf.* Still *et al.* (1)). The standard and richest source of the Krebs enzyme is pig kidney. The

aqueous extract of an acetone powder of pig kidney has been found to contain roughly equal amounts of the two oxidases (*cf.* Table IV). In his original study (4) Krebs reported that his preparations were virtually inactive towards D-aspartic acid. This result is probably due to the fact that the preparation of his acetone powder was not carried out at low temperatures and the D-aspartic oxidase was preferentially inactivated.

Inhibition of Split Oxidase by Benzoate—The non-identity of the D-aspartic oxidase and the D-amino acid oxidase has been based largely on the insensitivity of the former to benzoate, an inhibition which appears to be specific for the D-amino acid oxidase of Krebs. It was therefore important

TABLE IV
Ratio of Activity of D-Aspartic Oxidase to Krebs D-Amino Acid Oxidase in Pig and Sheep Kidney Preparations

Source of enzyme	Additions	Oxygen uptake	
		10 min.	20 min.
		μ l.	μ l.
Pig	None	20	38
"	DL-Methionine (100 μ M)	565	940
"	DL-Aspartate (100 μ M)	388	718
Sheep	None	16	32
"	DL-Methionine	560	820
"	DL-Aspartate	211	395

The enzymes were prepared from acetone powders of pig and sheep kidney minces (*cf.* the experimental section). The additions were 1.0 cc. of enzyme, 0.2 cc. of 10 per cent ethyl alcohol, substrate in the amount indicated. Total volume 3.0 cc. Air in gas space.

to know whether the split aspartic oxidase was also insensitive to the presence of benzoate. The velocity of oxidation of the split oxidase was found to be unaffected by benzoate.

EXPERIMENTAL

Preparation of Dissociated D-Aspartic Oxidase—Rabbit kidney or liver was homogenized in 5 volumes of cold 0.9 per cent potassium chloride in a Waring blender. The suspension was centrifuged, and the supernatant fluid was chilled and mixed with 8 volumes of acetone at -10° . The precipitate was filtered with suction, and the cake of precipitate washed thoroughly with acetone and then dried in air. The dry powder was rubbed up in 10 volumes of 0.1 M phosphate of pH 7.5 for 60 minutes. The insoluble residue was removed by centrifugation and the clear extract was mixed with 0.5 volume of saturated ammonium sulfate. All the aspartic oxidase activity was found in the precipitate formed at one-

third saturation of ammonium sulfate. The precipitate was taken up in the minimal volume of water and the solution was dialyzed 5 to 7 hours against running tap water and 12 hours against deionized water at 2°. It is to be noted that ammonium sulfate inhibits the D-aspartic acid oxidase and no tests of activity can be carried out in the presence of any considerable amounts of the salt. The resulting preparation was stable for several days when kept frozen at -10°.

SUMMARY

The D-aspartic oxidase is a conjugated flavoprotein which can be resolved into an apooxidase which is inactive unless supplemented with flavin-adenine dinucleotide.

This investigation was supported by a grant from the Commonwealth Fund.

BIBLIOGRAPHY

1. Still, J. L., Buell, M. V., Knox, W. E., and Green, D. E., *J. Biol. Chem.*, **179**, 831 (1949).
2. Ratner, S., Nocito, V., and Green, D. E., *J. Biol. Chem.*, **152**, 119 (1944).
3. Warburg, O., and Christian, W., *Biochem. Z.*, **298**, 368 (1938).
4. Krebs, H. A., *Biochem. J.*, **29**, 1620 (1935).

THE EFFECT OF FOLIC ACID ON RAT LIVER D-AMINO ACID OXIDASE*

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(Received for publication, September 20, 1949)

It has been previously reported that the administration of folic acid¹ to folic acid-deficient chicks brings about a very marked decrease in the xanthine oxidase activity of the livers of these animals (1). It seemed possible that this property of folic acid might be general for all flavin enzymes. For the present investigation the flavin enzyme D-amino acid oxidase was chosen because of its relative ease of measurement and because it has probably been the most thoroughly studied of the flavin enzymes.

The influence of folic acid and of injectable liver extract (LE) on D-amino acid oxidase activity of rat livers has been determined under normal conditions and under conditions of glycine toxicity and of hyperthyroidism. Glycine toxicity in rats has been shown to cause a growth depression which is almost completely overcome by the administration of folic acid (2). Experimental hyperthyroidism has been shown by Klein (3) to be accompanied by a marked elevation in the D-amino acid oxidase activity in the livers of rats and Martin (4) has shown that it increases the demand for folic acid. These conditions thus seemed suitable for the present investigation.

The results of this investigation indicate that there is a tendency toward a decrease in D-amino acid oxidase activity, under the conditions employed, when folic acid is present in the diet. This decrease, however, is not nearly as marked as that observed previously in chick liver xanthine oxidase.

EXPERIMENTAL

The animals used in these experiments were litter mate weanling rats of the Sprague-Dawley strain. The basal diet consisted of Labco casein

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¹ Pteroylglutamic acid.

18 gm., sucrose 75 gm., hydrogenated vegetable oil 3 gm., cod liver oil 2 gm., and salt mixture (5) 2 gm. To each kilo were added inositol 1 gm., choline chloride 1 gm., thiamine chloride 5 mg., riboflavin 5 mg., pyridoxine chloride 5 mg., calcium pantothenate 10 mg., nicotinic acid 20 mg., menadione 0.25 mg., and biotin 0.024 mg. The various modifications of this diet which were employed are given in Table I.

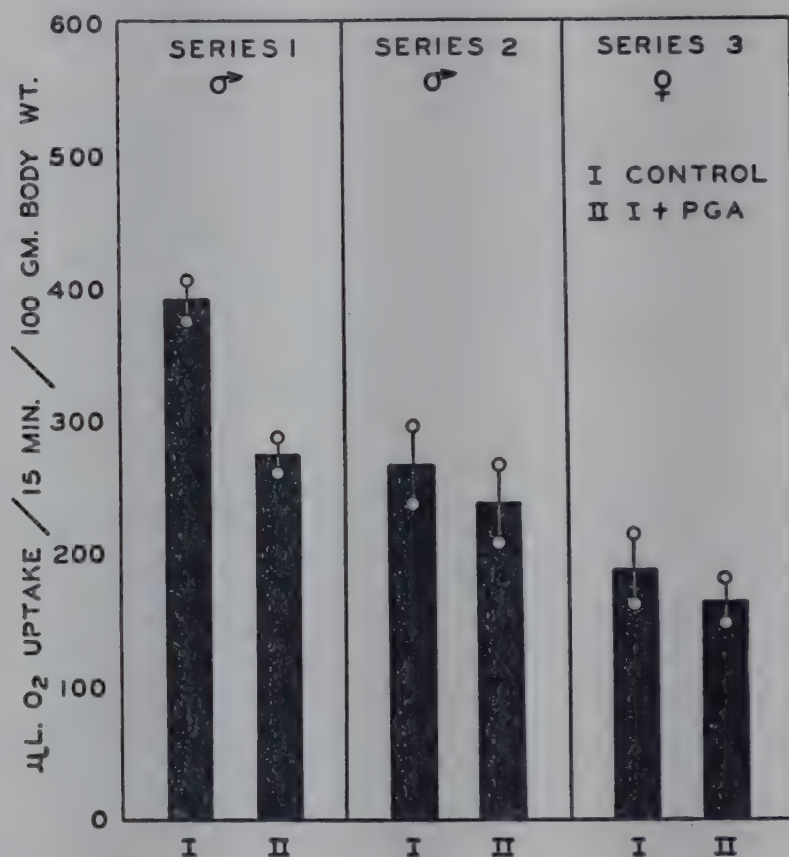


FIG. 1. Effect of folic acid on the D-amino acid oxidase activity of the livers of rats receiving a basal diet. The average values for O_2 uptake are represented by the bars and the standard error of the mean is represented by the circles. (PGA = folic acid).

After varying periods of time on the experimental diets, the animals were killed by decapitation and the livers removed. The livers were weighed and a 1:5 homogenate was prepared by use of a Waring blender. The determination of D-amino acid oxidase activity was carried out by measuring oxygen consumption in a Warburg apparatus. The flasks contained 1 ml. of tissue homogenate, 1.4 ml. of pyrophosphate buffer of pH 8.6, and 0.1 ml. of water; 0.5 ml. of 0.24 M DL-alanine was placed in the side arm and the center well contained 0.1 ml. of 10 per cent NaOH. The total volume in all cases was 3.1 ml. The contents of the side arm were tipped into the flasks immediately after the initial reading was taken. Manometer readings were taken at 15 minute intervals for 3 hours.

DISCUSSION AND RESULTS

Fig. 1 illustrates the effect of folic acid on the D-amino acid oxidase activity of the livers of rats receiving the basal diet. In Series 1 there was a significant decrease in activity when folic acid was added to the diet, but in Series 2 the differences in activity with and without folic acid were not statistically significant. There was a general trend in both series, however, toward a decrease in activity with the administration of folic acid. The animals in Series 1 had received the experimental diets

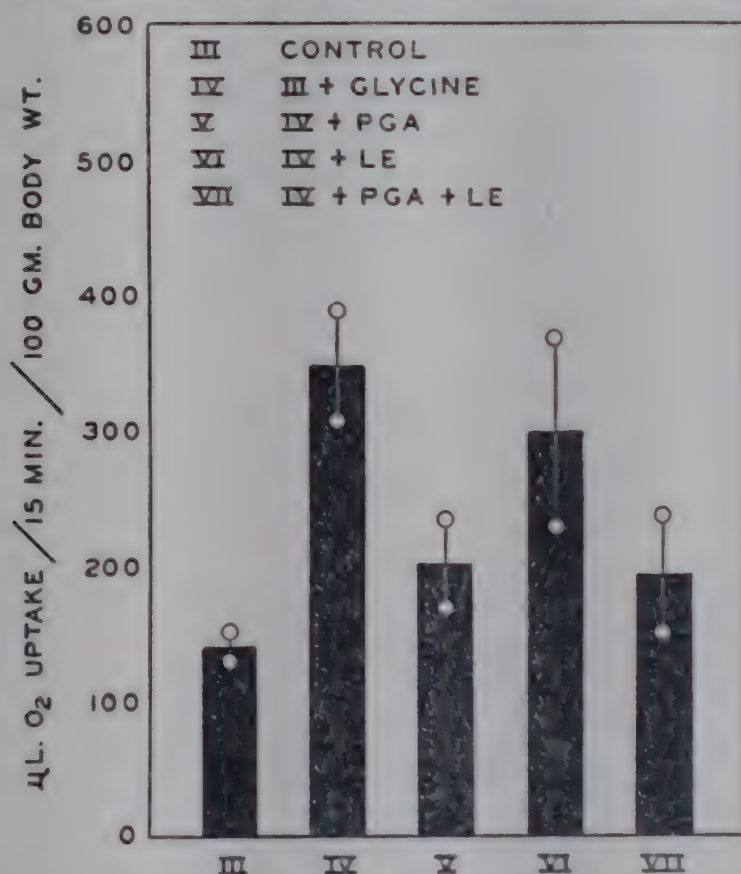


FIG. 2. Effect of folic acid and LE on the D-amino acid oxidase activity of the livers of rats receiving a diet high in glycine. The average values for O₂ uptake are represented by the bars and the standard error of the mean is represented by the circles. (PGA = folic acid).

for only 28 days, whereas those in Series 2 had received the diets for 50 to 60 days. Microbiological analysis of the folic acid content of these livers revealed that in Series 2 the animals receiving the unsupplemented basal diet had a relatively high folic acid content in the liver and thus a pronounced lowering of the D-amino acid oxidase activity might not be expected with the addition of folic acid to the diet. The results of Series 3 indicate that there is very little, if any, sex difference in the response of this enzyme system to folic acid administration.

The elevation of liver D-amino acid oxidase activity brought about by the inclusion of 10 per cent glycine in the basal diet and the effect of folic

acid and of LE in decreasing this activity are illustrated in Fig. 2. As can be seen from the weight gains of these animals (Table I), there was a definite impairment in the growth brought about by the presence of 10 per cent glycine which was almost completely overcome by the addition of folic acid and was alleviated to some extent by LE. A decrease in the D-amino acid oxidase activity per 100 gm. of body weight accompanied the increased growth observed with both the folic acid and the LE. The enzyme activity was expressed in terms of body weight, since the enzyme

TABLE I

Summary of Diet Compositions, Length of Experiments, and Weight Gains of Animals Receiving Purified Diets with Various Supplements

The glycine when added replaced an equal amount of sucrose. The folic acid was added at a level of 5 mg. per kilo of diet and the injectable liver extract*(LE), which contained 15 units per ml., was added at a level of 8 ml. per kilo of diet.

Group No.	No. of animals	Days on diet	Supplement to basal diet	Average total gain in weight during experimental period
				gm.
I. Series 1	6	28-30	None	131
" " 2	8	40-42	"	178
" " 3	16	32-38	"	117
II. " 1	6	28	Folic acid	136
" " 2	8	40-42	" "	173
" " 3	16	32-38	" "	119
III	3	50-60	None	162
IV	6	50-60	10% glycine	38
V	5	50-60	10% " + folic acid	131
VI	6	50-60	10% " + LE	75
VII	6	50-60	10% " + folic acid + LE	144

* Lederle Laboratories.

activity is probably related to the food intake which is in turn reflected in the weight of the animal. A similar experiment was carried out on female rats and the results were essentially the same as those obtained with the males. It appears that in glycine toxicity folic acid and to some extent LE are involved in the regulation of the activity of D-amino acid oxidase.

The observation that the addition of desiccated thyroid to the diet of rats brings about an increase in D-amino acid oxidase activity has been confirmed and the effect of folic acid and of LE on this increased activity has been determined (Table II). Since these animals received the experimental diets for a relatively short period, it seemed advisable to calculate

the O₂ uptake on the basis of change in weight as well as final body weight. The absolute values for O₂ uptake in Experiment II were approximately 50 per cent of those in Experiment I in all cases. The reasons for this are unknown, but it may possibly be due to the previous diet of the animals

TABLE II

*Effect of Folic Acid and of LE on D-Amino Acid Oxidase Activity of Livers of Rats Receiving Purified Diet Containing 0.7 Per Cent Thyroid Powder**

Experiment No.	Diet†	No. of animals	Gain in weight	1:5 Homogenate	Whole liver	
				Average O ₂ uptake per 15 min. per ml.	Average O ₂ uptake per 15 min. per 100 gm. change in weight	Average O ₂ uptake per 15 min. per 100 gm. body weight
			gms.	μl.	μl.	μl.
I	Basal (control)	5	81	14.2 ± 0.98‡	603 ± 15.5‡	377 ± 36.3‡
	" + thyroid	5	61	24.7 ± 3.60	1142 ± 209.4	634 ± 106.3
	" + " + 500 γ folic acid per 100 gm.	5	55	17.1 ± 2.63	809 ± 95.2	440 ± 64.6
	Basal + thyroid + 0.8 ml. LE per 100 gm.	5	58	23.8 ± 3.78	1186 ± 217.9	646 ± 114.5
	Basal + thyroid + 500 γ folic acid + 0.8 ml. LE per 100 gm.	4	66	22.7 ± 4.52	1099 ± 226.1	643 ± 161.7
II	Basal (control)	6	85	8.3 ± 1.64	368 ± 46.4	187 ± 40.6
	" + thyroid	7	43	14.9 ± 3.35	1014 ± 56.1	339 ± 75.4
	" + " + 500 γ folic acid per 100 gm.	4	44	9.5 ± 0.75	631 ± 35.1	215 ± 17.8
	Basal + thyroid + 0.8 ml. LE per 100 gm.	5	39	13.6 ± 2.97	1022 ± 151.6	305 ± 68.8
	Basal + thyroid + 500 γ folic acid + 8 ml. LE per 100 gm.	6	41	16.4 ± 3.49	1064 ± 201.0	376 ± 127.8

* Lilly's thyroid powder No. 58; iodine content 0.17 to 0.23 per cent.

† All animals received the basal diet for 7 days and then were placed on the various supplemented diets for 8 to 12 days.

‡ Standard error of mean.

or to the fact that the experiments were carried out at different times of the year. In both series there was a significant reduction in the D-amino acid oxidase activity with the addition of folic acid to the diet, and, as in the case of glycine toxicity, LE appeared to have no effect.

Contrary to the results obtained with glycine toxicity, LE given in combination with folic acid seemed to counteract the depression in activity noted when folic acid alone was fed.

SUMMARY

Data are presented which indicate that the D-amino acid oxidase activity of rat liver is decreased by the addition of folic acid to the diet of normal animals and to the diet of animals subjected to glycine toxicity and to experimental hyperthyroidism.

BIBLIOGRAPHY

1. Keith, C. K., Broach, W. J., Warren, D., Day, P. L., and Totter, J. R., *J. Biol. Chem.*, **176**, 1095 (1948).
2. Dinning, J. S., Keith, C. K., Totter, J. R., and Day, P. L., *Federation Proc.*, **8**, 381 (1949).
3. Klein, J. R., *J. Biol. Chem.*, **128**, 659 (1939).
4. Martin, G. J., *Am. J. Digest Dis.*, **14**, 341 (1947).
5. Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutr.*, **14**, 273 (1937).

THE IMMUNOLOGICAL DETERMINATION OF HUMAN ALBUMIN IN BIOLOGICAL FLUIDS

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The determination of serum albumin by an immunological method was first described by Goettsch and Kendall (1) in 1935 in dogs. Subsequently, the technique was applied to human serum (2-4). These early applications of the immunological technique were hampered by the difficulty in obtaining pure samples of human albumin for the preparation of antisera. Recent advances in the fractional precipitation of plasma proteins with alcohol have made large amounts of albumin available of sufficient purity to permit its use as an antigen. Such material was used by Chow (5) in the estimation of serum albumin. He showed good agreement with simultaneous electrophoretic determinations. Kabat, Glusman, and Knaub (6) have prepared antiserum to γ -globulin as well as to albumin from material obtained by alcohol fractionation, which proved useful in estimating these proteins in biological fluids.

In the present paper further evidence is presented for a close relationship between albumin concentrations determined immunologically and electrophoretically in sera with extreme differences in protein fractions. The application of the technique to a variety of biological fluids is demonstrated. In addition, the use of a new method for the quantitative estimation of protein in antigen-antibody precipitates is described. The method employs the ninhydrin reaction in a procedure similar to that of Moore and Stein for amino acid analyses (7).

Materials

Albumin Standard—The Red Cross concentrated human serum albumin produced by E. R. Squibb and Sons was used. This usually contained between 24 and 25 gm. of albumin per 100 cc.

Rabbit Antiserum—The above albumin solution was used for injecting rabbits in a manner similar to that described by Chow (5). Equal volumes of albumin, 5 mg. per cc. or 1 mg. per cc., and 0.5 per cent alum were used. The antiserum obtained was centrifuged prior to use. This was important even if the antiserum appeared clear (4).

The reagents used for the ninhydrin procedure were the same as those described by Moore and Stein (7).

Procedure

The method is carried out by diluting serum or other fluids with saline to give a final albumin concentration between 0.01 and 0.03 mg. per cc. A 0.5 cc. aliquot is placed in a small cuvette (10 × 75 mm.). To the cuvette 0.2 cc. of antiserum is then added and the mixture is allowed to stand for 1 hour at room temperature. At the end of this time the precipitate is handled in a manner similar to that described by Kabat and Mayer (4). Following centrifugation and pouring off of the supernatant with drainage on filter paper, 2 cc. of saline are added to the packed precipitate. This is broken up by rotating and shaking the tube and is recentrifuged. The procedure is repeated two more times, making a total of three washings with saline. The washed precipitate is dissolved in 0.1 cc. of NaOH (0.1 N), and 0.5 cc. of ninhydrin solution is added. The tubes are then placed in a boiling water bath for 20 minutes, after which 2 cc. of 1:1 water-propanol diluent are added. Following shaking, the tubes are read in a Coleman junior spectrophotometer at 570 m μ . A saline-antiserum mixture, carried through the entire procedure, is used as a blank.

A standard curve is prepared by using solutions containing 0.01, 0.02, and 0.03 mg. per cc. of albumin in place of serum in the above procedure. These dilutions were made up from a stock solution, the albumin of which had been determined by micro-Kjeldahl analysis with the nitrogen factor 6.25. The color produced with serum may be transferred directly to mg. of albumin from this standard curve.

EXPERIMENTAL

In carrying out a large number of albumin determinations by the immunological technique, the importance of conserving antiserum soon became apparent. The method as described by Chow (5) requires the use of 2 cc. of rabbit serum for each determination and, if these are done in duplicate or triplicate, 4 to 6 cc. of rabbit serum are needed. The phenol reagent as employed by Heidelberger and MacPherson (8) and the protein absorption in the Beckman spectrophotometer by the procedure of Eisen (9) and Gitlin (10) are known to be more sensitive methods of analyzing quantitatively the protein in antigen-antibody precipitates. A comparison of these various techniques, together with other methods of protein estimation, was therefore undertaken. Table I illustrates the approximate minimal amount of protein (albumin) that could be measured by various procedures under comparable conditions. The amount of protein that gave a final optical density reading of 0.07 in a volume of 2.5 cc. in the spectrophotometer was used for comparison. The direct measurement of protein in the Beckman spectrophotometer was carried out at a wave-length of 287 m μ in a solution of 0.1 N NaOH. The smallest

amounts of protein were detected by the ninhydrin procedure of Moore and Stein (7).

This method also has the advantage that it has been critically worked out to give a stable color and highly reproducible results with amino acids. The method is not specific for amino groups in proteins. Color is also given by peptides, amino acids, amines, and NH_3 . The presence in the precipitate of any of these substances except NH_3 is unlikely, and with minimal precautions contamination with NH_3 can be prevented.

Various proteins give somewhat different intensities of color with the ninhydrin reagent. Fig. 1 illustrates the comparison between albumin and an electrophoretically homogeneous γ -globulin preparation in respect to

TABLE I

Comparison of Sensitivity of Various Methods of Protein Estimation

The amount of protein giving a final optical density reading of 0.07 in the spectrophotometer is listed. The numbers in parentheses refer to the bibliography.

Method	Protein
	mg.
Ninhydrin (7).....	0.02
Colorimetric Kjeldahl (12).....	0.06
Biuret reagent (13).....	0.80
Beckman spectrophotometer (10).....	0.09
Phenol reagent (8).....	0.10
Micro-Kjeldahl*.....	1.20

* The figure listed for the micro-Kjeldahl is the minimal amount that could be conveniently titrated.

intensity of color at various nitrogen concentrations as determined by micro-Kjeldahl analysis. It is apparent that albumin gives a more intense ninhydrin reaction. However, for the analysis of the immune precipitate this difference offered no interference because of the constant ratio of albumin to antibody.

Although it was demonstrated by Chow (5) that albumin could be determined quantitatively in the zone of antibody excess, further experiments were carried out to determine the exact point where an approximate linear relationship between albumin concentration and precipitated protein ended. Varying quantities of pure albumin and also serum were tested with a constant amount of antiserum. The immune precipitate was estimated with the ninhydrin reagent. Fig. 2 illustrates the results for pure albumin and also for serum. The albumin content of this serum had been determined immunologically. The amount of antiserum used for each determination in this experiment was 0.3 cc. The curves for

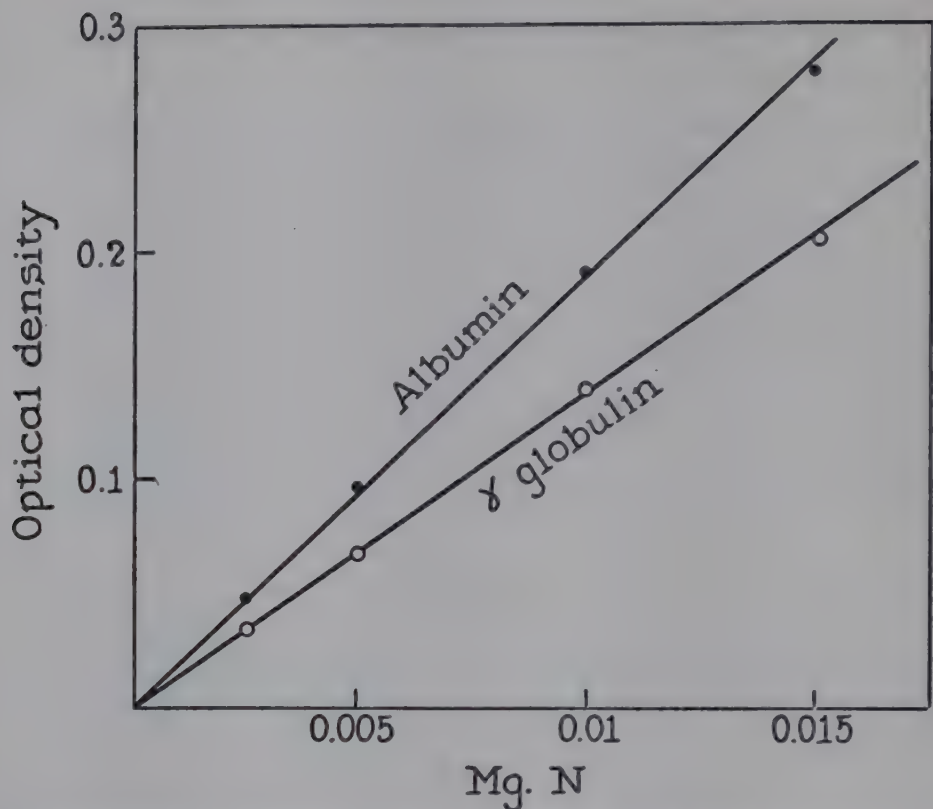


FIG. 1. Comparison of the intensity of color produced with ninhydrin by albumin and γ -globulin per unit of nitrogen.

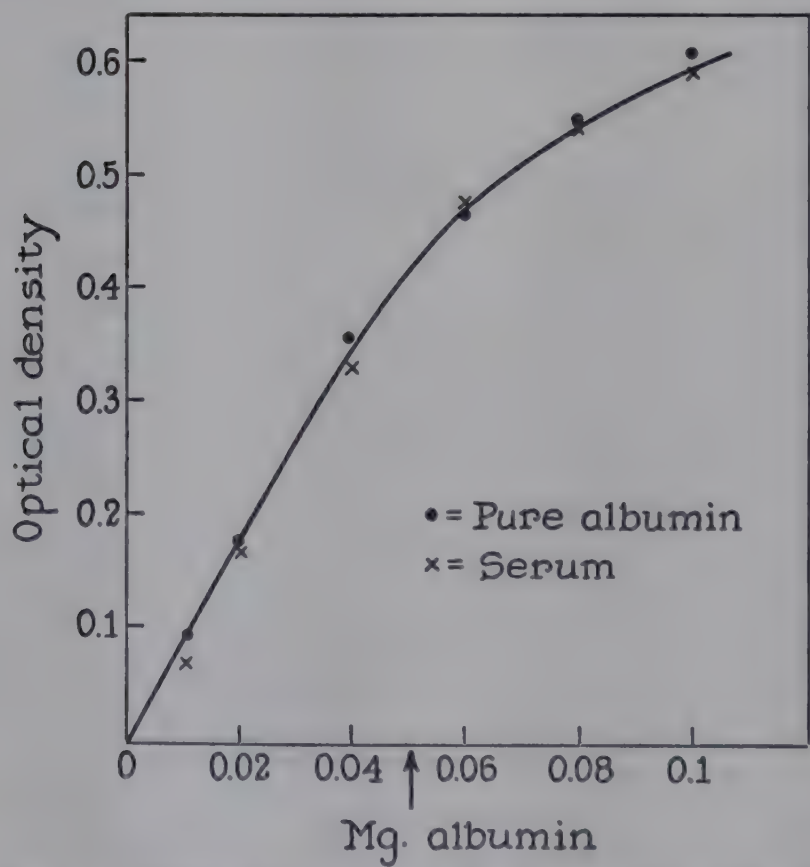


FIG. 2. Effect of increasing amounts of antigen (albumin and serum) on the quantity of antigen-antibody precipitate as measured with the ninhydrin reagent. The antibody concentration was kept constant.

the albumin and serum were completely parallel, leveling off above a concentration of 0.05 mg. of albumin in each case. There was no interference from the proteins other than albumin in the serum. The relationship between albumin concentration and immune precipitate approaches linearity below 0.05 mg. of albumin. Various batches of anti-serum showed slightly different critical levels for the ratio of albumin to antiserum below which this relationship held. However, if the ratio of mg. of albumin to cc. of antiserum was kept below 0.15, accurate quantitative results for albumin could always be obtained. In the experiments described in this report the ratio was kept below 0.1.

TABLE II

Variability of Albumin Values Obtained in Two Sera and One Ascitic Fluid with Three Dilutions of Antigen, Analyses in Triplicate

Serum or ascitic fluid	Albumin			Average
	Experiment 1	Experiment 2	Experiment 3	
cc.	gm. per cent	gm. per cent	gm. per cent	
0.0006	0.612	0.595	0.597	0.578 \pm 6%
0.0012	0.563	0.564	0.571	
0.0018	0.562	0.573	0.571	
0.0002	3.10	2.90	2.98	2.92 \pm 3%
0.0004	2.97	2.83	2.85	
0.0006	2.90	2.92	2.90	
0.0002	4.32	4.23	4.25	4.21 \pm 3%
0.0003	4.22	4.10	4.20	
0.0004	4.25	4.20	4.15	

Table II shows the results of three series of analyses on the same specimens of serum and ascitic fluid. Each analysis was carried out at three dilutions of antigen, all of which were below the critical level of antibody excess. The reproducibility of the results was slightly better at a single concentration of antigen, but even with the use of various dilutions of antigen the values fell within approximately ± 5 per cent of the average.

Incubation of the antigen-antibody mixture was not found to have any advantages over simple standing for 1 hour at room temperature. The correlation between various concentrations of serum and standard albumin solution was equally good at room temperature. In addition, standing at 0° for various periods after incubation did not offer any advantage. With antiserum which was not completely fresh, higher blanks were sometimes obtained after prolonged standing.

Electrophoretic Correlation—The immunological procedure for the determination of albumin was carried out on twenty-two sera and three

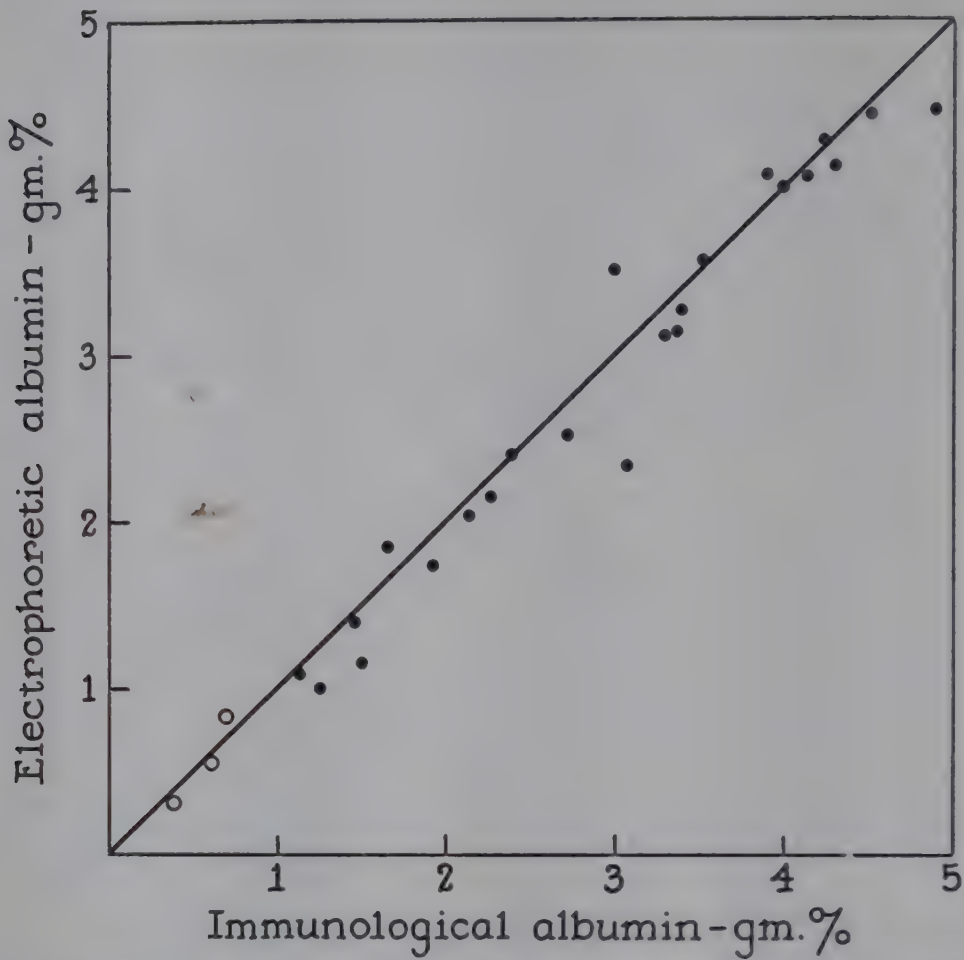


FIG. 3. Correlation between albumin concentrations determined immunologically and electrophoretically. ○ represents ascitic fluid.

TABLE III

Comparison of Electrophoretic and Immunological Determinations of Albumin in Sera of Widely Divergent Protein Composition

Total protein	Electrophoretic analysis		Immunological analysis
	γ-Globulin	Albumin	Albumin
	gm. per cent	gm. per cent	gm. per cent
2.3	0.3	1.1	1.2
4.3	0.6	2.1	2.3
7.6	1.0	4.4	4.9
9.2	6.1	2.5	2.8
11.4	7.1	3.1	3.6
12.1	7.2	3.6	3.4

ascitic fluids which previously had been subjected to electrophoretic analysis for other purposes.¹ Some of the sera had been stored at 0° for periods

¹ Sera with elevation in lipides were not used because of the increased error involved in correlating electrophoretic area with protein concentration.

of 6 to 18 months. No sera were used which showed evidence of protein sediment. Electrophoretic patterns were obtained by the method of Longsworth (11). The amount of albumin was calculated by multiplying the area of the albumin peak in per cent by the total protein as determined by micro-Kjeldahl analysis. The factor 6.25 was used for the calculation of protein from nitrogen, both for the electrophoretic albumin and for the standard curve of the immunological procedure. Fig. 3 illustrates the close correlation between the albumin determinations by the electrophoretic and immunological procedures. 75 per cent of the points fall below the line for perfect correlation, indicating slightly higher values for the immunological procedure. On averaging all the values, this difference amounts to 0.12 gm. per cent.

Table III lists the albumin determinations by the two methods in six sera of widely divergent protein composition. The concentration of γ -globulin is also listed, because this represented the chief abnormality in the high protein sera. The high γ -globulin sera were obtained from a group of patients with unusual liver disease. The correlation between the two albumin methods was similar, regardless of the concentration of other proteins.

DISCUSSION

The estimation of the quantity of antigen-antibody precipitate by the ninhydrin method of Moore and Stein (7) appeared to have certain advantages for measuring albumin over other methods now in use. Smaller quantities of antiserum were necessary, 0.2 cc. of antiserum or less depending on the potency of the antiserum. As little as 3 γ of albumin could be accurately measured in biological fluids. The method was more sensitive than that of Heidelberger and MacPherson (8) utilizing the phenol reagent and that of Gitlin (10) employing absorption in the Beckman spectrophotometer. The color produced with ninhydrin was more stable than that with the phenol reagent.

Measurements of albumin concentration were carried out in serum, ascitic fluid, chest fluid, urine, and tissue extracts. The method proved of particular value when the concentration of albumin was particularly low and when albumin determinations by the Howe method would have little significance. Electrophoretic determinations are also difficult under such conditions because of the necessity of concentrating the material prior to analysis. In urines, for example, this often means concentration of pigments which interfere with the determinations.

Comparison of albumin levels in sera of widely divergent protein concentrations by the immunological and the electrophoretic methods showed a good correlation. The correlation was equally good at the low albumin

levels existing in ascitic fluid. Electrophoretic determinations were not carried out on urines, but the results would be expected to be similar to those in ascitic fluids.

SUMMARY

The reaction of ninhydrin with NH_2 groups has been utilized as the basis for a photometric determination of protein in antigen-antibody precipitates. Comparison with common methods of protein determination indicated that this was more sensitive. The procedure permitted the use of smaller quantities of antiserum for the determination of albumin in biological materials. The correlation between the immunological and the electrophoretic methods of determining albumin was good regardless of the concentration of albumin and other proteins. Slightly higher values for albumin were obtained by the immunological procedure. The method proved particularly useful for determinations of small quantities of albumin in biological fluids other than serum.

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BIBLIOGRAPHY

1. Goettsch, E., and Kendall, F. E., *J. Biol. Chem.*, **109**, 221 (1935).
2. Goettsch, E., and Reeves, E. B., *J. Clin. Invest.*, **15**, 173 (1936).
3. Goettsch, E., and Lyttle, J. D., *J. Clin. Invest.*, **19**, 9 (1940).
4. Kabat, E. A., and Mayer, M. M., *Experimental immunochemistry*, Springfield (1948).
5. Chow, B. F., *J. Biol. Chem.*, **167**, 757 (1947).
6. Kabat, E. A., Glusman, M., and Knaub, V., *Federation Proc.*, **7**, 306 (1948); *Am. J. Med.*, **4**, 653 (1948).
7. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **176**, 367 (1948).
8. Heidelberger, M., and MacPherson, C. F. C., *Science*, **97**, 405 (1943).
9. Eisen, H. N., *J. Immunol.*, **60**, 77 (1948).
10. Gitlin, D., *J. Immunol.*, **62**, 437 (1949).
11. Longworth, L. G., *Chem. Rev.*, **30**, 323 (1942).
12. Wong, S. Y., *J. Biol. Chem.*, **55**, 431 (1923).
13. Weichselbaum, T. E., *Am. J. Clin. Path.*, **7**, 40 (1946).

A SPECIFIC QUALITATIVE COLOR TEST FOR KETOHEXOSES

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Ketohexoses (fructose and sorbose) or compounds which yield ketohexoses (sucrose, raffinose, and inulin) give rise to a bright reddish purple color in the presence of aminoguanidine and sulfuric acid. The color is stable for several hours. Equivalent amounts of aldohexoses produce no color in 24 hours.

In this reaction guanidine nitrate or nitroguanidine cannot be used instead of aminoguanidine; therefore the amino group attached to the guanidine molecule probably is essential to the formation of the colored compound.

The following pentoses, aldohexoses, and aldohexose-yielding compounds produce no color under similar conditions: arabinose, xylose, ribose, glucose, galactose, mannose, lactose, maltose, melibiose, dextrin, starch, and glycogen. No color is given by formaldehyde or by the pure proteins, crystalline pepsin, crystalline trypsin, and crystalline chymotrypsin. Furfural gives a yellow color and furfuryl alcohol a brown color. Acetone, methylethyl ketone, and levulinic acid produce a very slight yellow color.

EXPERIMENTAL

Aminoguanidine Solution—2.5 gm. of aminoguanidine sulfate \cdot H₂O (Eastman Kodak Company, No. 4023) in 100 cc. of distilled water. This solution is very stable at room temperature.

Sulfuric Acid—Concentrated sulfuric acid, c.p.

Color Test

0.5 cc. of concentrated sulfuric acid is placed in a 12 \times 100 mm. test-tube. 0.2 cc. of the aminoguanidine sulfate solution is added without mixing. 0.2 cc. of the test solution containing 0.4 mg. of fructose, sorbose, or inulin is added, and the contents of the tube are well mixed. A bright reddish purple color forms in about 1 minute and increases in intensity for some time. 0.4 mg. samples of aldohexoses gave no color in 24 hours. A color identical to that given by 0.4 mg. of fructose, sorbose, or inulin is obtained with 0.8 mg. of sucrose or 1.2 mg. of raffinose.

As small an amount as 50 γ of fructose may be readily detected by this test. Solutions containing as much as 2 mg. of aldohexose per test pro-

duce a hardly noticeable light lilac color. More concentrated aldohexose solutions, however, produce a color similar to the one given by the dilute ketohexose solutions. This interference is probably caused by the conversion of a small quantity of the aldohexose into the color-forming compound by the sulfuric acid. Thus solutions which are to be tested for ketohexose should be diluted to contain not more than 3 mg. of total carbohydrate per cc. or 0.6 mg. per test.

It is important to note that the reagents and carbohydrate solutions should be exactly measured in order to eliminate possible reactions by aldohexoses.

SUMMARY

A convenient and rapid color reaction for ketohexoses and conditions for obtaining specificity with the test have been described.

A QUANTITATIVE IMMUNOCHEMICAL STUDY OF FERRITIN AND ITS RELATION TO THE HEPATIC VASODEPRESSOR MATERIAL*

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The identity of ferritin or its iron-free protein, apoferritin, with a hepatic vasodepressor material (VDM) has been reported previously (1). This factor, together with an oppositely acting vasotropic principle (VEM, vasoexcitor material) of renal origin, has been revealed as a regular participant in hemorrhagic shock and in experimental renal (dog) and essential (human) hypertension. Further studies (2-4) have revealed the presence of VDM in the plasma of rats suffering from nutritional cirrhosis, in the plasma of patients with hepatic cirrhosis, heart failure, and toxemia of pregnancy, as well as in the edema fluid in nephrosis and congestive heart failure. Ferritin was shown to exert a marked antidiuretic effect in dogs and rabbits (5), a finding which correlates with its presence in those conditions associated with antidiuresis and edema.

The identity of ferritin with VDM was established for the VDM produced by normal dog liver on anaerobic incubation, for the VDM present in the saline wash of liver slices from dogs in the hyporeactive or irreversible stage of hemorrhagic shock, for the VDM present in the plasma of dogs in the irreversible stage of hemorrhagic shock, and for the VDM present, together with VEM, in the plasma during the chronic stage of experimental renal (dog) and essential (human) hypertension. Ferritin was identified by direct isolation in crystalline form from extracts of aerobic liver slices. Identification of ferritin with the VDM present in the conditions cited above was made by a combined immunochemical procedure and the rat mesoappendix test of Zweifach and Chambers (6). The vasodepressor effect of these hepatic extracts and plasma samples could be abolished specifically by prior incubation with rabbit antiserum to the homologous crystalline ferritin.

The present report gives the results of a study of the quantitative immunochemical behavior of ferritin and apoferritin. These data provided

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the basis for ascertaining the relationship of ferritin to the vasodepressor principle, VDM, in various biological fluids in which the concentration is too low for direct isolation or analysis. These immunochemical characteristics were also utilized for the study of the chemical properties of ferritin and apoferritin and for the direct quantitative estimation of ferritin in various tissues of the dog.

Methods

Preparation of Protein Antigens—Crystalline ferritin and apoferritin were prepared as described previously (1). They were recrystallized four

TABLE I
Protein Preparations Used As Antigens

Antigen	Source	Preparation No.	Fe:N ratio <i>mg. Fe per mg. N</i>
Ferritin	Horse spleen	E1162-3*	1.88
	" "	HSF67	1.54
	" "	E1160	1.57
	" "	HSF4	1.31
	" liver	HoLF1	1.22
	Dog liver	PE1164	1.06
	" "	DLF2	1.24
	Human liver	HLF10	1.17
	Horse spleen	HSAF2†	0.0
VDM	Dog liver	DSL B	1.17
	Horse liver	HLVD-B	0.78
	" "	HLVD-A	0.88
	" "	HLVD-C	0.28

* On a dry weight basis, N 11.0, Fe 20.7, P 1.29, Cd 0.23 per cent.

† On a dry weight basis, N 16.2, Fe 0.0, P 0.05, Cd 0.16 per cent.

times with CdSO_4 , reprecipitated four times at 50 per cent saturation with $(\text{NH}_4)_2\text{SO}_4$ in order to reduce the Cd content of the protein, and dialyzed against running tap water overnight. Repeated treatment of ferritin with $\text{Na}_2\text{S}_2\text{O}_4$ and α, α' -dipyridyl, according to Granick (7), was necessary in order to obtain apoferritin completely free of iron.

The hepatic VDM fractions studied were prepared during the course of a fractionation of liver extracts by methods designed to concentrate its vasodepressor properties as measured by the rat mesoappendix test. Table I lists the iron-nitrogen ratios of all such protein preparations used in the present study.

Bioassay for VDM Activity—The vasodepressor activity of solutions containing either crude VDM concentrates, ferritin, or apoferritin, was

assayed by the rat mesoappendix test of Zweifach and Chambers (6) in the following manner. Prior to injection of the test material, the reactivity was determined of the terminal muscular vessels (the metarterioles and precapillaries) in the exteriorized mesentery of an anesthetized rat to the topical application of a threshold concentration of epinephrine. The test rat was then injected intravenously with 0.5 ml. of the test solution and the effect noted on the reactivity of the terminal vessels. The injection of a solution with vasodepressor activity will depress or completely abolish the responsiveness of these vessels to the original threshold concentration of epinephrine for a period of from 20 to 40 minutes. This degree of inhibition is similar to that which follows the injection of 0.5 ml. of dog plasma obtained during the hyporeactive or irreversible phase of hemorrhagic shock. The injection of 0.5 ml. of normal dog plasma will not alter the response to epinephrine. These bioassays were conducted under the supervision of our associate, Dr. B. W. Zweifach.

*Preparation of Antiserum and Quantitative Precipitin Reaction*¹—Rabbit antiserum to crystalline ferritin or apoferritin was obtained as previously described (1). Twelve rabbits were used for each series of immunizations and those rabbits chosen for pooling of sera whose preliminary antibody titers were highest. Good titers of antibody were obtained by using a total of 30 mg. of alum-precipitated antigen, injected intravenously during a period of 4 weeks. Blood was withdrawn for the preparation of serum 5 days after the last injection. Subsequent injections of antigens were given in order to maintain or increase the antibody content of the rabbit serum. The chilled antisera were preserved with 1:10,000 merthiolate and centrifuged in the cold to remove any particulate or lipid matter. In certain instances several antisera were pooled, partially purified, and concentrated by means of two successive precipitations of the "globulin" fraction at 50 per cent saturation with $(\text{NH}_4)_2\text{SO}_4$, followed by exhaustive dialysis against 0.9 per cent saline. A fixed quantity of antiserum (1 ml.) was treated with appropriate increments of antigen solution and enough 0.9 per cent saline to make a final volume of 3 ml. After incubation at 37° for 1 hour and 48 hours in the refrigerator, the precipitates were centrifuged in the cold, washed twice with 3 ml. portions of cold saline, and analyzed for total nitrogen. The first supernatant was divided. One portion was tested with antigen and the other with antiserum in order to determine the presence of excess antibody or antigen. The equivalence zone was that portion of the precipitin curve in which the supernatants showed neither antibody nor antigen in excess.

For certain experiments the quantitative precipitin reaction was carried

¹ We are indebted to Dr. E. A. Kabat, Neurological Institute, New York, for his advice and many details concerning the quantitative precipitin technique.

out on a micro scale, with quantities of added antigen N as low as 1 γ . In these experiments, the precipitation was performed in conical centrifuge tubes, and the mixtures were incubated for 1 hour at 37° and placed in the refrigerator for 7 to 10 days with occasional mixing. The quantity of washed antibody-antigen complex precipitated was estimated by the use of the Folin color reagent as adapted by Heidelberger and MacPherson (8).

Chemical Analyses—Total nitrogen was determined by a modified micro-Kjeldahl method (9) with 100 ml. digestion flasks. The ammonia was steam-distilled into 5 ml. of saturated boric acid containing 1 drop of a mixed indicator, methylene blue-methyl red. The ammonia was titrated directly with standard 0.01 N HCL. Iron was determined colorimetrically (10) with a Klett-Summerson photocolormeter after digestion with sulfuric acid and treatment with potassium persulfate and KCNS. Total phosphorus was determined colorimetrically (11). Cadmium was determined according to Granick and Michaelis (7). The protein content was estimated by a dry weight determination of accurate aliquots of the solution.

EXPERIMENTAL

Electrophoretic Studies on Ferritin and Apoferritin—Although no one method is available for the absolute determination of the purity of proteins, the value of electrophoresis for this purpose is well recognized. The extent of homogeneity of the crystalline ferritin and apoferritin preparations which were used as antigens in this study was determined by a series of electrophoretic analyses.² Apoferritin was studied at a concentration of 1 per cent, whereas ferritin solutions were examined at concentrations of 0.2 to 0.5 per cent, due to the extensive light absorption of the deep brown ferritin solution. Uniform single boundaries were obtained with no evidence of any impurities with differing mobilities. Table II gives the values for the mobilities of horse ferritin and apoferritin at various pH values. It may be seen that the mobilities of these two proteins are essentially the same from a pH of 4.0 to 8.6. The isoelectric point of horse ferritin, as well as of horse apoferritin, estimated from these data is 4.4. Thus, the presence of over 20 per cent by weight of iron in ferritin does not affect the charge on the protein molecule. Single boundaries have also been obtained for the dog and human ferritin preparations used in these studies, with higher isoelectric points of 5.2 and 5.5 respectively.

Immunochemical Identity of Ferritin and Apoferritin—In view of the identical mobilities of ferritin and apoferritin and the identical vasode-

² These analyses were carried out in collaboration with Dr. R. F. Furchgott, Greta Landwehr, and Barbara Gottlieb in our laboratory. We are indebted to Dr. V. du Vigneaud for the use of the electrophoresis apparatus.

TABLE II

Electrophoretic Mobilities of Horse Spleen Ferritin and Apoferritin

The determinations were carried out with a Tiselius-Longsworth apparatus on solutions of 0.2 to 0.5 per cent ferritin and 1.0 per cent apoferritin. The mobilities are for ascending boundaries. The ferritin was Preparation E1162-3 and the apoferritin, Preparation HSAF2. Ionic strength, 0.1.

pH	Buffer	Mobility, cm. ² per volt-sec. $\times 10^6$	
		Ferritin	Apoferritin
3.95	Acetate	+1.9	+1.9
4.5	"	-0.6	-0.5
5.3	"	-3.3	-3.3
7.3	Phosphate	-6.3	-6.2
8.6	Veronal	-6.1	-6.2

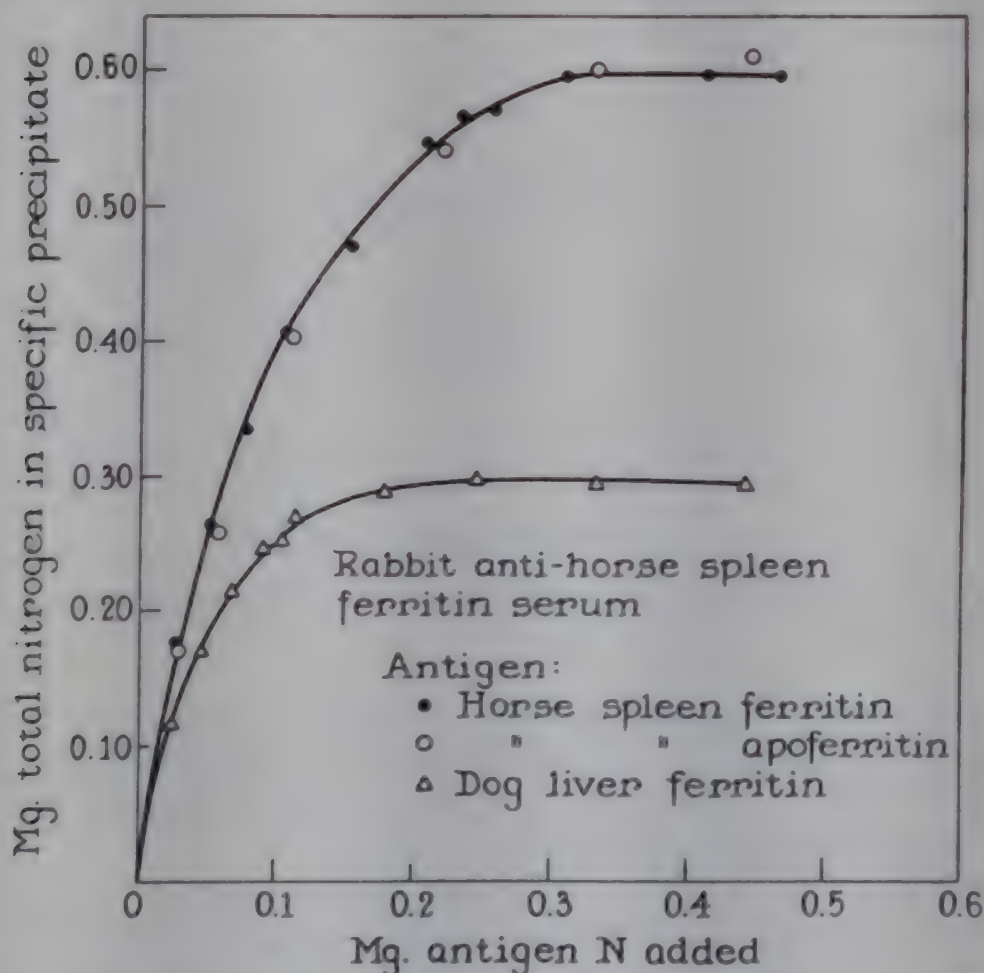


FIG. 1. Quantitative precipitin curves for rabbit antiserum to crystalline horse spleen ferritin.

pressor activities of these two proteins as measured by the rat meso-appendix test, it was of interest to compare them immunochemically by means of the quantitative precipitin method. Horse spleen ferritin was

injected into a series of rabbits and the antiserum thus obtained studied with respect to its ability to precipitate known quantities of horse spleen ferritin and apoferritin. The topmost curve of Fig. 1 shows that the same quantity of total nitrogen in the specific precipitate was obtained whether the antigen used was ferritin or apoferritin. The antibody produced in response to immunization with ferritin was therefore directed towards the protein moiety of ferritin. The topmost curve of Fig. 2 complements the above results, since it was obtained by using a rabbit antiserum to horse

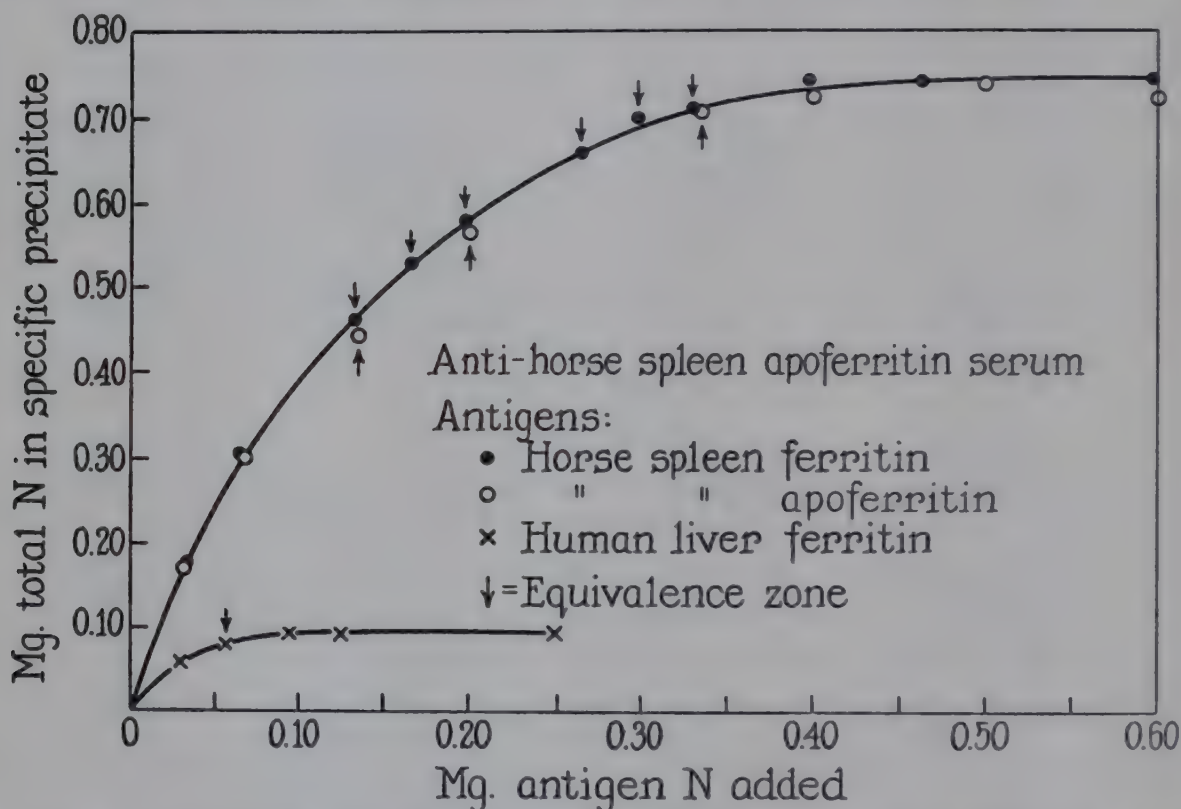


FIG. 2. Quantitative precipitin curves for rabbit antiserum to crystalline horse spleen apoferritin.

spleen apoferritin. The antibody to apoferritin reacted identically, in the quantitative precipitin test, towards apoferritin and ferritin; the values for the total nitrogen in the specific precipitates were on the same curve when plotted against either ferritin or apoferritin.

Participation of Ferritin Iron in Precipitin Reaction—In order to determine whether the iron of ferritin was quantitatively precipitated along with the protein moiety, experiments were carried out in such a manner that both the total nitrogen and total iron content of the specific precipitates could be determined. Antiserum to horse spleen apoferritin was partially purified by precipitation of the "globulin" fraction at 50 per cent saturation with $(\text{NH}_4)_2\text{SO}_4$ and dialyzed against 0.9 per cent saline to remove the last traces of $(\text{NH}_4)_2\text{SO}_4$. For each value of ferritin nitrogen

added, quadruplicate tubes were prepared. Two of these were analyzed for the total nitrogen and the other two for the total iron in the washed specific precipitates. Each supernatant obtained after the first centrifugation of the precipitate was tested for the presence of excess antibody or antigen. Table III lists the results obtained. In the zone of excess antibody, as well as in the equivalence zone, in which all the added antigen had been precipitated, all of the iron added in the form of ferritin was quantitatively recovered in the specific precipitate. In the zone of excess antigen, in which some antigen remained in the supernatant, the amount of

TABLE III

Participation of Ferritin-Iron in Precipitin Reaction

The antibody was a "globulin" fraction from a rabbit antiserum to crystalline horse spleen apoferritin (Preparation HSAF2), diluted with saline. The antigen was crystalline horse spleen ferritin (Preparation E1162-3). Analyses for total nitrogen and total iron in the precipitates were performed in duplicate.

Added antigen		Recovered in ppt.		Recovery of iron	Excess in supernatant
N	Fe	N	Fe		
mg.	mg.	mg.	mg.	per cent	
0.033	0.057	0.178	0.058	102	Antibody
0.066	0.114	0.305	0.111	97	"
0.132	0.228	0.463	0.225	99	"
0.165	0.285	0.529	0.283	99	Equivalence zone
0.198	0.342	0.579	0.350	102	" "
0.264	0.456	0.660	0.445	98	" "
0.297	0.513	0.700	0.520	101	" "
0.330	0.570	0.711	0.570	100	" "
0.396	0.684	0.743	0.635	93	Antigen
0.462	0.798	0.743	0.640	80	"
0.594	1.026	0.748	0.645	63	"

iron recovered in the precipitate was less than the total iron added in the form of ferritin. Thus, the iron of ferritin is quantitatively precipitated along with the protein moiety, and is attached to the protein in such a way that it does not interfere with the specific combination of antibody with antigen. These results permitted the calculation of the quantity of antibody N in the specific precipitates in the zone of excess antibody and in the equivalence zone by subtracting the added antigen N from the total N in the precipitate. Also, from the iron content of the precipitates it was possible to calculate the antibody N content of the precipitates in the zone of excess antigen.

Molecular Composition of Specific Precipitates—Calculations of the molecular composition of various specific precipitates obtained with rabbit

antiserum and homologous antigens have been made by Heidelberger (12). The molecular weight of crystalline horse apoferritin has been determined by Rothen (13) using the ultracentrifuge and was found to be 465,000.

TABLE IV
Molecular Composition of Specific Precipitates from Rabbit Antisera

Antigen	Mol. wt.	Molecular ratio, antibody to antigen		
		Extreme anti-body excess zone	Equivalence zone	
			Antibody excess side	Antigen excess side
Horse serum albumin*	67,000	6	4	3
“ apoferritin	465,000	26	14	7
Thyroglobulin*	700,000	40	14	10

* Data taken from Heidelberger (12).

TABLE V
Inhibition of Precipitin Formation by Excess Antigen

Rabbit antiserum to horse spleen ferritin was treated with increasing quantities of horse spleen ferritin, Preparation E1160.

Ferritin N added	Total N precipitated	Tests on supernatant	
<i>mg.</i>	<i>mg.</i>		
0.026	0.176	Excess antibody	
0.051	0.265	“	“
0.077	0.335	“	“
0.102	0.407	“	“
0.153	0.473	Equivalence zone	
0.204	0.548	“	“
0.230	0.568	“	“
0.255	0.571	Excess antigen	
0.306	0.596	“	“
0.408	0.595	“	“
0.459	0.597	“	“
1.232	0.598	“	“
1.642	0.570	“	“
2.053	0.541	“	“
2.464	0.533	“	“
4.106	0.377	“	“
5.338	0.243	“	“

The molecular weight of the rabbit antibody protein was taken as 150,000 (14). With use of the values for antibody nitrogen precipitated at various points on the precipitin curve, the molecular composition of the antibody-apoferritin complex was obtained and is recorded in Table IV. For

purposes of comparison, similar ratios for the horse serum albumin and the thyroglobulin systems are also given, as examples of proteins with lower and higher molecular weights than ferritin.

Inhibition of Precipitin Formation by Excess Antigen—The egg albumin antibody-antigen system (15) is an example of the inhibition of precipitin formation in the presence of excess antigen. In this region of the precipitin reaction, soluble compounds of antibody and antigen are formed which remain in the supernatant, together with uncombined antigen, so that

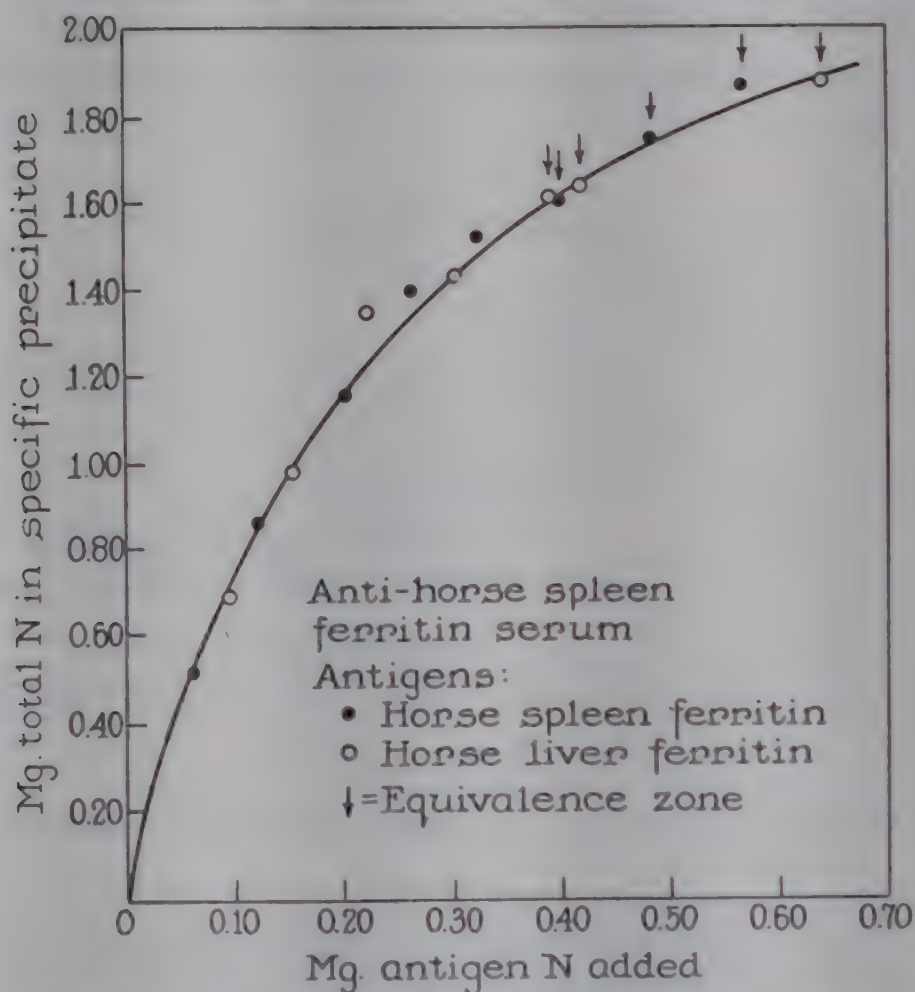


FIG. 3. Quantitative precipitin curves obtained with horse liver and spleen ferritins added to a rabbit antiserum to crystalline horse spleen ferritin.

precipitation is less than maximal. Table V gives the results of the addition of relatively large amounts of horse spleen ferritin to a rabbit antiserum to horse spleen ferritin. With increasing amounts of added antigen the amount of total N precipitated decreased, although a fairly broad zone of maximal precipitation in the region of excess antigen was observed.

Immunochemical Identity of Horse Spleen and Horse Liver Ferritins—The quantitative precipitin reaction was applied to the problem of the identity of horse spleen and liver ferritins. These two ferritins yield crystals with CdSO_4 which are indistinguishable. Fig. 3 shows the pre-

cipitin curve obtained when horse liver and spleen ferritin were added to a rabbit antiserum to horse spleen ferritin. The values for the total nitrogen in the washed specific precipitates obtained with these liver and spleen ferritins fall on the same curve. These two ferritins are therefore immunochemically identical.

Presence and Nature of Ferritin in Dog Liver VDM Concentrates—Our previous report (1) presented evidence of the identity of ferritin with hepatic VDM based on a number of similarities: identical crystals with CdSO_4 , identical visible light absorption spectra, correlation of vasodepressor activity with iron content, and finally the pronounced vasodepressor

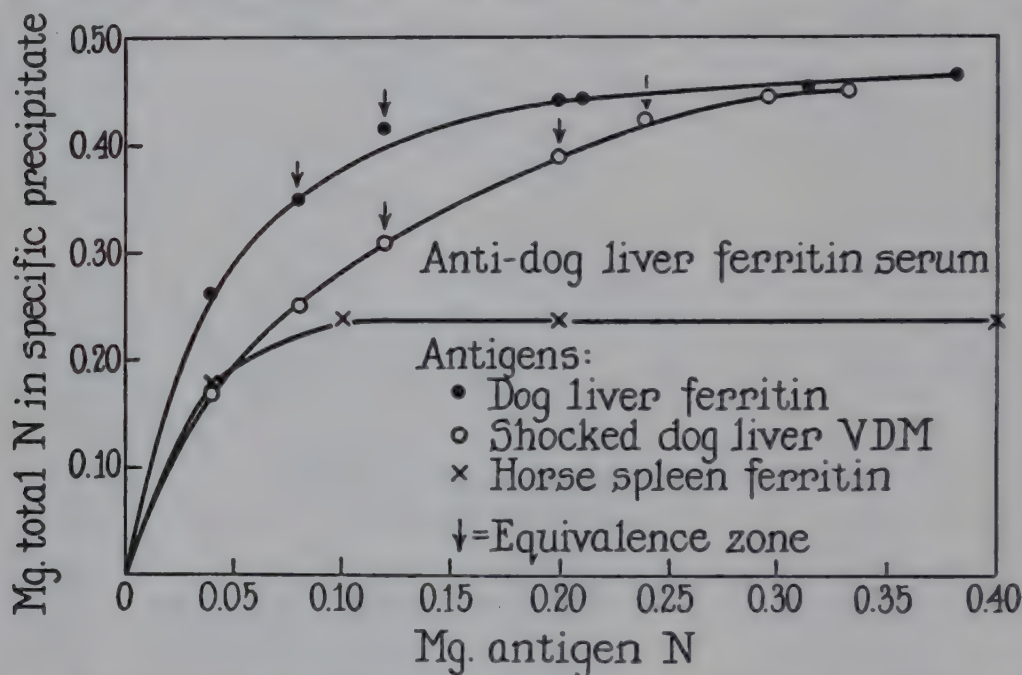


FIG. 4. Quantitative precipitin curves for rabbit antiserum to crystalline dog liver ferritin.

activity of crystalline ferritin which was indistinguishable from that of VDM concentrates. In order to identify the iron-protein component in the VDM solutions more specifically with ferritin, the quantitative immunochemical method was applied. A solution of recrystallized dog liver ferritin was compared, immunochemically, with a preparation of dog liver VDM which had been fractionated so as to achieve increasing vasodepressor activity, as measured by the rat mesoappendix test. Fig. 4 shows the precipitin curves obtained with a rabbit antiserum to crystalline dog liver ferritin. The curves show that, with equal quantities of ferritin nitrogen and of dog liver VDM nitrogen, the total nitrogen in the precipitate obtained with ferritin was greater than that found with the VDM concentrate. As a consequence, the early portions of the curves are not superimposable. However, when increasing amounts of dog liver VDM were added, up to the maximal precipitating capacity of the antiserum, maximal precipi-

tation of total nitrogen was obtained at a level corresponding to that obtained with ferritin. Thus, the dog liver VDM solution contained ferritin and, in addition, some non-ferritin protein which was not precipitable by the antiserum. The quantitative data permit a calculation of the per cent ferritin present in the dog liver VDM solution (16). Table VI lists a comparison of the relative amounts of ferritin and VDM nitrogen required to obtain identical amounts of total nitrogen in the specific precipitates in the zone of excess antibody. From this calculation, ferritin nitrogen comprised 52 per cent of the total nitrogen in the dog liver VDM preparation. Vasodepressor activity of the dog liver ferritin was obtained at 0.0005 γ of N, whereas the dog liver VDM concentrate gave equivalent

TABLE VI

Per cent Ferritin in Dog Liver VDM from Immunochemical Data (See Fig. 5)

The VDM activity of the dog liver ferritin (Preparation DLF2) was obtained with 0.0005 γ of nitrogen per 0.5 ml., that for the dog liver VDM (Preparation DSLB) with 0.001 γ of nitrogen per 0.5 ml. The values for the total nitrogen in the specific precipitates were chosen so as to correspond to the portion of the curves where antibody is present in excess.

Total nitrogen in ppt.	Antigen N of ferritin	Antigen N of VDM	Per cent ferritin in VDM
mg.	mg.	mg.	
0.300	0.056	0.112	50
0.320	0.066	0.130	51
0.360	0.087	0.170	51
0.380	0.100	0.190	53
0.400	0.115	0.212	54
Average.....			52

vasodepressor activity with 0.001 γ of N. Thus, there was good correspondence between the immunochemical and biological data.

Fig. 4 also shows the precipitin curve obtained by the addition of horse ferritin to rabbit antiserum to dog ferritin. Maximal precipitation occurred at a much lower level of total nitrogen in the specific precipitates than with dog ferritin. These results indicate that the ferritins of these two species are immunologically related but not identical.

Ferritin in Horse Liver VDM Solutions—Results similar to those in the preceding section have also been obtained with horse liver VDM solutions compared immunochemically with horse ferritin (1). Fig. 5 presents the results of additional experiments which illustrate the identity of the iron-protein component in VDM concentrates with ferritin. A horse liver VDM concentrate had been prepared, during the course of fractionation studies, which contained 0.78 mg. of Fe per mg. of N. A portion of this solution

was fractionated with varying concentrations of $(\text{NH}_4)_2\text{SO}_4$. The fraction precipitated at 29 per cent saturation with $(\text{NH}_4)_2\text{SO}_4$ contained 0.88 mg. of Fe per mg. of N. Another fraction, precipitated between 32 and 50 per cent saturation with $(\text{NH}_4)_2\text{SO}_4$, contained 0.28 mg. of Fe per mg. of N. Each of the three fractions was used as an antigen and added to a rabbit antiserum to crystalline horse ferritin and the quantitative precipitin curves compared with a curve obtained with crystalline horse ferritin as an antigen. The results show a correlation between the Fe:N ratio of the VDM solutions and the closeness of its precipitin curve to that of crystalline

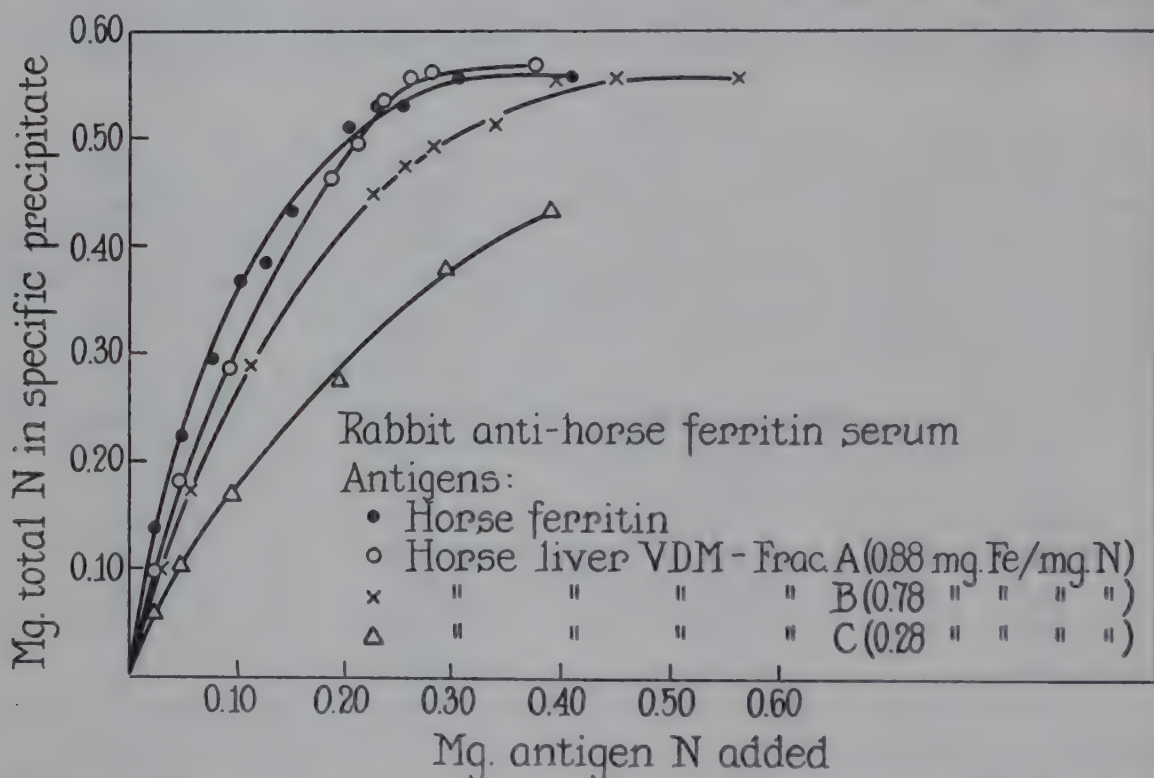


FIG. 5. Quantitative precipitin curves obtained with various subfractions of horse liver VDM added to a rabbit antiserum to crystalline horse spleen ferritin.

ferritin. Furthermore, those VDM fractions which contained 0.88 and 0.78 mg. of Fe per mg. N, respectively, reached a point of maximal precipitation of total N equal to that observed with ferritin. In the case of the VDM fraction with 0.28 mg. of Fe per mg. of N insufficient material was available to complete the remainder of the curve.

Immunochemical Reactions of Human Ferritin—Horse spleen is the only source of crystalline ferritin from which a relatively good yield can be obtained. Human ferritin is difficult to obtain, due to its source and the low yields. It was of interest, therefore, to determine whether a cross-reaction exists between horse and human ferritin so that antiserum to horse ferritin could be used in experiments with human ferritin. Fig. 6 shows the quantitative precipitin curves obtained for an antiserum to human liver

ferritin when treated with human liver ferritin and with horse spleen ferritin. A definite cross-reaction exists. Fig. 2, which gives the curves obtained for an antiserum to horse spleen apoferritin, shows the cross-reaction with human liver ferritin. Evidence for a positive cross-reaction between dog and human ferritin was also obtained. 1 ml. of a rabbit antiserum to crystalline dog liver ferritin was treated with a slight excess of various ferritins so as to yield a maximal precipitation of total N in the washed specific precipitates. Addition of dog liver ferritin yielded 0.33 mg. of total N, horse spleen ferritin 0.15 mg. of total N, and human liver ferritin 0.08 mg. of total N in the precipitates obtained.

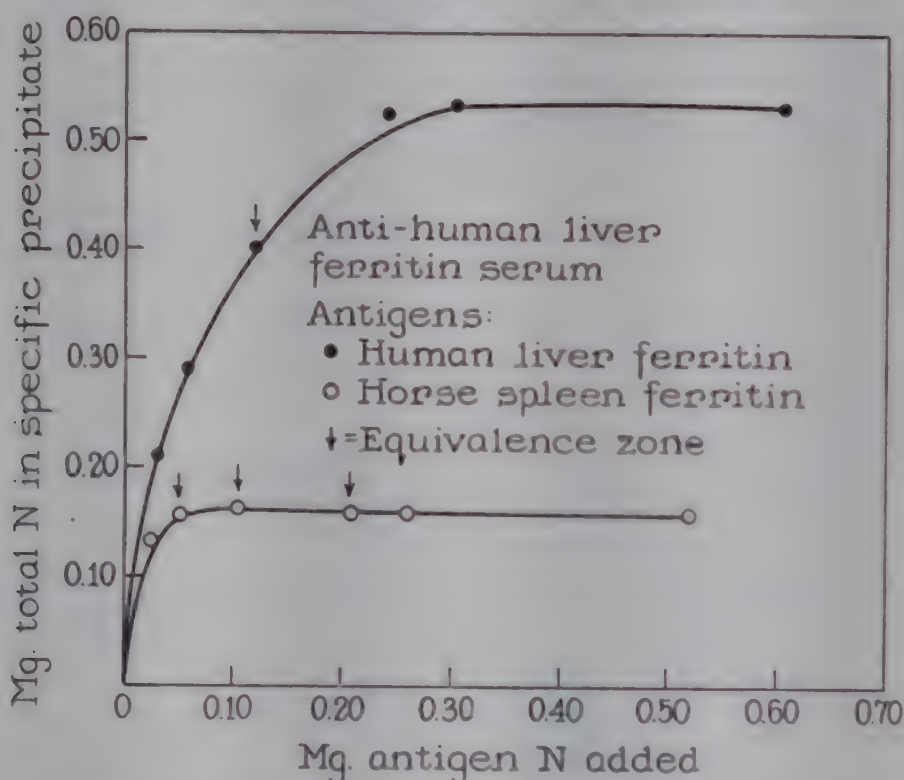


FIG. 6. Quantitative precipitin curves for rabbit antiserum to human liver ferritin

Inactivation of VDM Activity by Specific Antisera—Since crystalline ferritin gave a positive VDM test by the rat mesoxappendix technique at a level of 0.0005 γ of N per 0.5 ml. injected, it appeared that the VDM of shocked dog plasma, if identical with ferritin, would be present in a concentration too low to yield a visible precipitate when treated with antiserum to dog ferritin. In order to establish the identification of ferritin with the VDM from biological fluids such as plasma, a combination of the immunochemical and rat test procedures was employed. Table VII gives some typical results obtained by the *in vitro* incubation of ferritin or plasma containing VDM activity with homologous rabbit antiserum, followed by the subsequent injection of these mixtures into test rats. Normal rabbit serum or rabbit antiserum alone was without effect. Normal rabbit serum

incubated with ferritin or with a plasma having VDM activity did not affect the original response induced by the test sample alone. However, when ferritin or plasma with VDM activity was incubated with its homologous antiserum, the VDM response was abolished. This inactivation occurred with a concentration of antiserum equivalent to 0.08 ml. per 0.5 ml. of sample. A further series of tests was carried out to determine the extent of dilution of the antiserum which could inactivate 0.0005 γ of ferritin

TABLE VII
Inactivation of VDM Activity by Antiserum in Vitro

All mixtures were incubated at 37° for 30 minutes. 0.5 ml. of the mixture was injected for bioassay, sterile saline being used as a diluent. Homologous antisera were used for each ferritin or plasma. Dog plasma was obtained from animals in the hyporeactive or irreversible stage of hemorrhagic shock (26). Human plasma was from subjects with essential hypertension.

Mixture injected into test rat	Concentration of ferritin or plasma per 0.5 ml. injected	Concentration of rabbit serum per 0.5 ml. injected	Vasotropic effect
		ml.	
Normal rabbit serum (NRS).....		0.08	None
Rabbit antiserum (RAS).....		0.08	"
Ferritin + saline.....	0.0005 γ N		VDM
" + NRS.....	0.0005 " "	0.08	"
" + RAS.....	0.0005 " "	0.08	None
Shocked dog plasma + saline.....	0.33 ml.		VDM
" " " + NRS.....	0.33 "	0.08	"
" " " + RAS.....	0.33 "	0.08	None
Hypertensive human plasma + saline...	0.33 "		"
" " " + NRS....	0.33 "	0.08	"
" " " + RAS....	0.33 "	0.08	VEM*

* Hypertensive human plasma usually contains both VDM and VEM in a ratio such that their opposing vasotropic effects nullify each other in the rat test. Hence, the appearance of VEM activity is attributable to the inactivation of the VDM originally present.

N. With one rabbit antiserum to ferritin, the VDM response of 0.0005 γ of ferritin N was inactivated by the equivalent of 0.008 ml. of antiserum. Table VII also gives the results of incubation of human hypertensive plasma with rabbit antiserum to human liver ferritin. It had previously been shown in our laboratory (17) that the plasma of human subjects with chronic essential hypertension usually gave neutral results when injected into test rats, and that this was the consequence of the presence of a mixture of both VDM and VEM in high and approximately neutralizing amounts. The incubation of such plasma with rabbit antiserum to human ferritin

resulted in an inactivation of the VDM component and an unmasking of the VEM.

Table VIII shows the results of a study of the *in vivo* inactivation of ferritin and plasma VDM by the intravenous injection of rabbit antiserum to ferritin 1 hour prior to injection of the test sample. Results similar to those obtained in Table VII are shown. The results suggest that the injected antiserum is effective, *in vivo*, in inactivating VDM.

Complement Fixation by Ferritin Antibody-Antigen Complex—It was of interest to determine whether the ferritin antibody-antigen complex was capable of combining with complement. Experiments were carried out to

TABLE VIII

Inactivation of VDM Activity by Antiserum in Vivo

The substance to be tested was injected approximately 1 hour after the first injection of saline or rabbit serum. The hypertensive dog plasma was obtained from animals made chronically hypertensive by removal of one kidney and partial clamping of the remaining renal artery (27).

Material injected 1 hr. prior to injection of test substance	Test substance injected	Vaso-tropic effect in test rat
Saline, 0.5 ml.	Ferritin, 0.0005 γ N per 0.5 ml.	VDM
Normal rabbit serum, 0.5 ml. of 1:10	" 0.0005 " " " 0.5 "	"
Rabbit antiserum, 0.5 ml. of 1:10	" 0.0005 " " " 0.5 "	None
Saline, 0.5 ml.	Shocked dog plasma, 0.5 ml.	VDM
Normal rabbit serum, 0.5 ml. of 1:10	" " " 0.5 "	"
Rabbit antiserum, 0.5 ml. of 1:10	" " " 0.5 "	None
Saline, 0.5 ml.	Hypertensive dog plasma, 0.5 ml.	"
Normal rabbit serum, 0.5 ml. of 1:10	" " " 0.5 "	"
Rabbit antiserum, 0.5 ml. of 1:10	" " " 0.5 "	VEM

determine the smallest quantity of antigen (ferritin or apoferritin) which could be detected by this method. The complement fixation tests were run in a manner described by Kabat and Mayer (18) with two 100 per cent units of guinea pig complement, a 1:16 dilution of rabbit antiserum to ferritin or apoferritin, the antigen (ferritin or apoferritin) in varying concentrations, and sheep red cells sensitized with two units of hemolysin. Evidence of hemolysis was determined after a light centrifugation of the tubes and the color of the supernatant as well as the size of the red cell pellet used to estimate the completeness of hemolysis. In the horse, dog, and human ferritin antibody-antigen systems, 0.1 γ of ferritin or apoferritin N gave complete inhibition of hemolysis, whereas 0.05 γ gave a 2+ hemolysis. Suitable anticomplementary and hemolytic controls for antibody and antigen were negative. Positive complement fixation was

also obtained by cross-reacting antibody and antigen, *e.g.* antiserum to horse ferritin with dog ferritin.

Ferritin Content of Dog Tissues — The microprecipitin method (8) was used to determine the ferritin content of various tissues from normal dogs. Advantage was taken of the fact that ferritin solutions can be heated to 80° without apparent denaturation. Homogenates of tissues were prepared in the Waring blender with use of 5 volumes of water per gm. of wet tissue and the mixture heated to 78° for 10 minutes in a water bath. The cooled mixture was filtered and dialyzed against running water overnight. The clear filtered solution was adjusted to pH 7.4 and NaCl added to 0.9 per cent, together with merthiolate to a final concentration of 1:10,000. 10 ml., or a smaller aliquot which had been diluted with saline to 10 ml., were

TABLE IX

Ferritin Content of Dog Tissues by Microprecipitin Method

The following tissues gave negative results, indicating the presence of less than 0.5 γ of ferritin N per gm. of wet tissue: whole blood, kidney medulla, intestinal mucosa, and smooth muscle of the intestine.

Dog No.	Hemo- globin	R. B. C. $\times 10^6$	Ferritin N, γ per gm. wet tissue						
			Liver	Spleen	Bone marrow	Kidney cortex	Pan- creas	Skeletal muscle	Cardiac muscle
	<i>gm. per cent</i>								
4	16.2	6.1	134	90	16	18	6	2	1
5	14.1	6.2	65	99	18		8	5	1
6	14.1	6.1	75	166	12	14	3	9	2
7	14.1	6.1	115	42		22	7	2	1

treated with 1 ml. of a rabbit antiserum to dog liver ferritin and the mixture incubated for 1 hour at 37° and kept for 7 days in the refrigerator. A control was prepared with use of the same quantity of tissue extract treated with 1 ml. of normal rabbit serum. The washed precipitates were treated as described by Heidelberger for estimation of the Folin color value. Any color produced in the control was subtracted from the experimental value. The ferritin content of the precipitates was estimated from a standard curve obtained in a similar manner, with known amounts of dog liver ferritin added to 1 ml. of antiserum. That portion of the standard curve was used which corresponded to a large excess of antibody. This was almost a straight line. By this technique, 0.2 γ of ferritin N per ml. (2.0 γ of N per 10 ml.) yielded a precipitate that gave a color value of 130 when read on the Klett-Summerson photocolormeter against a reagent blank, with use of a No. 66 filter.

The results for several dogs are listed in Table IX. It may be seen that

ferritin was present in the liver, spleen, bone marrow, kidney cortex, skeletal muscle, cardiac muscle, and pancreas. None could be detected in whole blood, kidney medulla, intestinal mucosa, or smooth muscle of the intestine. The specific precipitates obtained with all but the pancreas were deep red in color, typical for ferritin of high iron content. Those obtained with pancreas were much paler. Iron analyses of the precipitates confirmed these observations, yielding an iron-nitrogen weight ratio for the ferritin from liver, spleen, marrow, kidney cortex, and muscles of from 2.0 to 2.5, whereas the ratio for pancreas was less than 1.0. Two other tissues, brain and lung, were also treated in a similar manner, but the tissue extracts were too viscous for analysis or yielded a precipitate when allowed to stand alone. In several experiments ferritin was found to be present in extracts from ovaries and testes, as shown by the color of the precipitate after addition of antiserum. However, quantitative values are not recorded, since, in these cases, the method of extraction produced viscous solutions or solutions which precipitated protein on standing alone in the absence of antiserum, thus yielding high blanks with normal rabbit serum.

DISCUSSION

Ultracentrifuge studies by Rothen (13) have shown that solutions of crystalline ferritin which had been prepared by the CdSO_4 method of Granick represented a mixture of 20 to 26 per cent apoferritin, together with ferritin, the latter in an aggregated state. He also showed that, when such a ferritin preparation was freed of its iron by reduction with $\text{Na}_2\text{S}_2\text{O}_4$ to yield apoferritin, the latter behaved as a single molecular species in the ultracentrifuge. Indications of the identity of these two proteins are the similar crystal forms obtained with CdSO_4 as well as similar x-ray data for ferritin and apoferritin (19). Yet, ferritin, crystallized with CdSO_4 , can be fractionated either with varying concentrations of $(\text{NH}_4)_2\text{SO}_4$ or by high speed centrifugation to yield fractions with a different ratio of Fe to N (20).

Our concern with the similarities of these two proteins stems from the fact that both ferritin and apoferritin, as well as the fractions obtained by $(\text{NH}_4)_2\text{SO}_4$ or high speed centrifugation, are equally active, on the basis of N content, in the rat mesoappendix test (6). Further, apoferritin is as active as ferritin in its ability to produce a marked antidiuretic effect when injected into dogs or rabbits (5). Other evidences for the probable similarity of these two proteins have been obtained from the quantitative amino acid data (20), as well as from the results presented here for the electrophoretic mobilities and the quantitative precipitin reactions. The electrophoresis data indicate that the total charge on the protein surface of ferritin is the same as that for apoferritin over a wide pH range, despite

the presence of over 20 per cent by weight of iron in ferritin. More recently we have mixed ferritin with apoferritin, crystallized such a mixture with CdSO_4 , and found a single electrophoretic boundary for this substance, with a mobility equal to that previously obtained for each when determined separately at that pH. The isoelectric point of horse ferritin of 4.4 is consistent with the analytical data for the acidic and basic amino acid content of this species of ferritin. Crystalline human ferritin has an isoelectric point of 5.5 and dog ferritin of 5.2.

The immunochemical data support the identity of ferritin and apoferritin with regard to surface-reactive groupings. However, it may be pointed out that immunochemical technique does not always provide absolute proof of such identity, since it has been shown (21) that a chemically altered protein (partially deaminated) may exhibit a precipitin reaction curve identical with that given by the unaltered protein. In our studies, immunochemical identity was suggested by the similarity in the antibody-antigen reaction when ferritin and apoferritin were allowed to react as antigens with the antibody of either antiferritin or antiapoferritin serum.

Heidelberger had proposed in his early studies of the quantitative precipitin reaction that, when an antigen was added to an antibody solution, a quantitative precipitation of the added antigen was obtained in those regions of the precipitin curve in which the supernatant was negative for excess antigen, namely, in the zone of excess antibody and in the equivalence zone. This view was criticized by Taylor, Adair, and Adair (22). Heidelberger supported his proposal by results obtained with azo dye-labeled proteins (23) and thyroglobulin (24), by analysis for the dye and organically bound iodine respectively, in the specific precipitates. In our studies, the high iron content of ferritin and the ease and accuracy of its determination enabled us to confirm the findings of Heidelberger. Table III clearly shows the quantitative recovery of iron in the specific precipitates in those regions of the precipitin curve when antigen was not present in excess. Further, since the quantity of total N precipitated by identical amounts of ferritin N or apoferritin N is the same, it may be concluded that the iron in ferritin does not interfere with or take part in the antibody-antigen reaction.

Others have used the quantitative precipitin reaction to estimate the quantity of a protein present in various body fluids (25). A prerequisite for such analytical methods is the availability of a reasonably pure protein antigen for the preparation of a specific antiserum and for establishing a standard curve of reference. The ferritin and apoferritin used in these studies were crystalline and homogeneous electrophoretically. By means of such a technique, the identity of the iron-protein in VDM liver concentrates with ferritin was established both for horse liver (1) and in this

study for dog liver. Reference to the standard curve also permitted calculation of the ferritin content of these crude liver concentrates and yielded values which agreed well with the vasodepressor activities of such extracts when compared with the vasodepressor activity of pure ferritin.

The combination of ferritin or apoferritin with its specific antibody results in a complete inactivation of the vasodepressor effect as measured by the rat mesoappendix test, even when the amounts of ferritin or apoferritin are too small to yield a visible precipitate. This finding enabled us to identify the VDM present in the plasma of man and animals in various pathological states with ferritin or apoferritin. This was accomplished by the inactivation of the vasodepressor effect in such plasma by specific antiserum to ferritin. In all cases, control incubation with normal rabbit serum did not affect the vasodepressor response of the test material. In this manner, the VDM present in the plasma of dogs with chronic renal hypertension and in human subjects with essential hypertension was shown to be due to ferritin or apoferritin. The value of this procedure for the identification of vasodepressor substance in various biological fluids is that it throws light on this identity in a manner otherwise impossible, since in such fluids we are dealing with amounts too small for any direct chemical estimation, especially in the presence of relatively large amounts of non-ferritin protein. The micro-precipitin reaction, as well as the complement fixation test, when applied to plasma giving a vasodepressor response, was negative, indicating that such plasma contained less than 0.05 γ of ferritin or apoferritin N per ml.

The calculation of molar ratios for antibody and antigen of ferritin gives a value of 26:1 in the region of great excess of antibody. When the quantitative precipitin reaction is utilized on a micro scale and analyses of the precipitate carried out by the Folin phenol reagent, very small quantities of antigen are needed for quantitative studies. Such a technique was used to determine the ferritin content of various dog tissues. These preliminary studies have established the wide distribution of ferritin in the animal organism as well as the variations in its concentration in various tissues, particularly liver and spleen. The variations in the latter organs may be a reflection, in large measure, of the state of iron metabolism in the animal. The wide distribution of ferritin in tissues apparently not involved in hematopoiesis may possibly indicate a relationship between the iron of ferritin and such compounds as cytochrome *c* and other heme catalysts. This analytical method is being utilized for the analysis for ferritin in dog tissues in various pathological states associated with altered iron metabolism and disturbances in peripheral circulation.

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SUMMARY

Immunochemical studies have been carried out on crystalline ferritin and apoferritin which have been identified as the hepatic vasodepressor material (VDM).

Ferritin and apoferritin were found to have identical electrophoretic mobilities over a wide pH range (4.0 to 8.6). This suggests the presence of iron in the ferritin molecule in such an arrangement as not to affect the surface charge. The following isoelectric points were determined for ferritins of different species: horse spleen ferritin and apoferritin, 4.4; dog liver ferritin, 5.2; human liver ferritin, 5.5.

Horse spleen ferritin and apoferritin were indistinguishable by the quantitative precipitin test. This was also true for the ferritins from liver and spleen of the horse.

The iron of ferritin is quantitatively precipitated along with the protein moiety when it is allowed to react with antiserum to ferritin. Since ferritin and apoferritin are indistinguishable immunochemically, the iron does not appear to affect the antibody-antigen reaction.

The previous observation that horse ferritin was identical with the vasodepressor material, VDM, obtained from horse liver under anaerobic conditions has been confirmed and extended. A similar identification was established for dog liver ferritin with VDM formed by anaerobic dog liver.

Immunochemical cross-reactions exist for horse, dog, and human ferritins, but the quantitative aspects of these cross-reactions show them to be different immunochemically.

By a combination of immunological procedures and direct visualization of vascular effects in the exteriorized omentum of the rat, the VDM activity exhibited by plasma in a variety of circulatory disturbances in animals and man was shown to be due to the ferritin content. By means of the microprecipitin technique, modified to permit the determination of ferritin in dog tissues, this substance was found to be present in liver, spleen, kidney cortex, bone marrow, pancreas, skeletal muscle, and cardiac muscle, in order of decreasing content. None could be detected in whole blood, kidney medulla, intestinal mucosa, or smooth muscle.

BIBLIOGRAPHY

1. Mazur, A., and Shorr, E., *J. Biol. Chem.*, **176**, 771 (1948).
2. Shorr, E., Zweifach, B. W., and Furchgott, R. F., *Science*, **102**, 489 (1945).
3. Shorr, E., Zweifach, B. W., Furchgott, R. F., and Baez, S., *Tr. Assn. Am. Physn.*, **60**, 28 (1947).

4. Baez, S., Zweifach, B. W., Mazur, A., and Shorr, E., *Proc. Soc. Exp. Biol. and Med.*, **64**, 154 (1947).
5. Baez, S., Mazur, A., and Shorr, E., *Federation Proc.*, **7**, 5 (1948); **8**, 7 (1949).
6. Chambers, R., Zweifach, B. W., Lowenstein, B. E., and Lee, R. E., *Proc. Soc. Exp. Biol. and Med.*, **56**, 127 (1944).
7. Granick, S., and Michaelis, L., *J. Biol. Chem.*, **147**, 91 (1943).
8. Heidelberger, M., and MacPherson, C. F. C., *Science*, **97**, 405 (1943); **98**, 63 (1943).
9. Kabat, E. A., and Mayer, M., *Experimental immunochemistry*, Springfield, 286 (1948).
10. Wong, S. Y., *J. Biol. Chem.*, **77**, 409 (1928).
11. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
12. Heidelberger, M., *J. Am. Chem. Soc.*, **60**, 242 (1938).
13. Rothen, A., *J. Biol. Chem.*, **152**, 679 (1944).
14. Kabat, E. A., and Pedersen, K. O., *Science*, **87**, 372 (1938). Kabat, E. A., *J. Exp. Med.*, **69**, 103 (1939).
15. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, **62**, 697 (1935).
16. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, **55**, 555 (1932). Heidelberger, M., and Kabat, E. A., *J. Exp. Med.*, **63**, 737 (1936). Kabat, E. A., and Glusman, M., *Am. J. Med.*, **4**, 653 (1948).
17. Shorr, E., Zweifach, B. W., Furehgott, R. F., and Beaz, S., *Federation Proc.*, **6**, 200 (1947).
18. Kabat, E. A., and Mayer, M., *Experimental immunochemistry*, Springfield, 109 (1948).
19. Fankuchen, I., *J. Biol. Chem.*, **150**, 57 (1943).
20. Mazur, A., and Shorr, E., *Federation Proc.*, **8**, 228 (1949).
21. Maurer, P., and Heidelberger, M., *Federation Proc.*, **8**, 227 (1949).
22. Taylor, G. L., Adair, G. S., and Adair, M. E., *J. Hyg.*, **34**, 118 (1934).
23. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, **62**, 467 (1935).
24. Stokinger, H. E., and Heidelberger, M., *J. Exp. Med.*, **66**, 251 (1937).
25. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, **62**, 697 (1935). Goettsch, E., and Kendall, F. E., *J. Biol. Chem.*, **109**, 221 (1935).
26. Zweifach, B. W., Lee, R. E., Hyman, C., and Chambers, R., *Ann. Surg.*, **120**, 232 (1944).
27. Goldblatt, H., Lynch, J., Hanzal, R. F., and Summerville, W. W., *J. Exp. Med.*, **59**, 347 (1934).

THE EXTRAHEPATIC SYNTHESIS OF CHOLESTEROL*

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Isotopic studies have clearly demonstrated the importance of the liver for cholesterol synthesis in the mammalian organism. In rats that had been fed deuterium-labeled acetate for 8 days, Bloch and Rittenberg found that the isotopic concentration of liver cholesterol was significantly greater than that of carcass cholesterol (1). This observation has been confirmed recently by Anker, with C^{14} -labeled acetate (2). Bloch and Rittenberg have estimated that the rate of cholesterol synthesis by the rat liver is sufficient to fulfil the cholesterol requirement of that animal (3).

But accumulated evidence in this laboratory shows that extrahepatic tissues can synthesize cholesterol. This evidence is presented here.

EXPERIMENTAL

Operative Techniques; Evisceration and Functional Hepatectomy—The rat was anesthetized with ether and its peritoneal cavity opened by a mid-ventral incision. The intestines were retracted and the celiac axis and the superior mesenteric arteries tied with a single ligature at their origin from the aorta. Double ligatures were next placed around the descending colon and inferior mesenteric artery. The latter courses along the middle of the colon. Double ligatures were then placed around the esophagus and the blood vessels adjacent to the region where the esophagus runs into the cardiac region of the stomach. The portal vein was next ligated. The animal's viscera were removed by cutting between the double ligatures and by severing the mesenteric attachments of the viscera to the body wall.

After removal of the viscera, the peritoneal cavity was packed with saline-soaked gauze. The muscular and cutaneous layers of the abdominal wall were then separately closed with interrupted sutures.

Adrenalectomy and Ovariectomy—These were carried out as described by Griffith and Farris (4).

In Vivo Procedures—The normal rats were injected subcutaneously, with an isotonic saline solution containing $C^{14}H_3C^{14}OONa$, at the beginning of the experiment and at 2 and 4 hours later. Each rat received a total volume of 4.5 cc.

* Aided by a grant from the United States Public Health Service.

The eviscerated rats also received three injections, each of which contained 300 mg. of glucose in addition to the labeled acetate.

In Vitro Procedures—Slices of liver, kidney, and adult brain were prepared free-hand. In the testis experiments, the tubules were teased apart after the capsule was removed. The brain of the new born rat was not sliced but was minced with a razor blade.

Skin was removed from the day-old rat as follows: The animal was first decapitated and the limbs amputated. Incisions were then made ventrally and dorsally along the length of the torso. The two sheets of skin obtained were cut into pieces measuring approximately 5 to 10 sq. mm.

In the case of the adult rat, areas of shaved skin were raised and severed from the body in the region of the loose areolar tissue. Pieces measuring 10 to 15 sq.mm. were used in the baths.

Cleaned small intestine was cut along its length and each longitudinal sheet then cut transversely to provide pieces approximately 10 to 20 sq. mm.

Chemical Procedures—Whole rats were hydrolyzed on a steam bath for 8 hours in an alcoholic KOH solution which was 30 per cent KOH and 50 per cent ethyl alcohol. 2 cc. of this solution were used for each gm. of body weight. The contents of the incubation flasks were also transferred to an alcoholic KOH solution, prepared so that after the addition of the incubation mixture the final concentration was 17 per cent KOH and 50 per cent ethyl alcohol; hydrolysis was carried out for 8 hours on a steam bath. After the hydrolysis the alcohol was allowed to evaporate. Cholesterol was then extracted according to the method of Sperry *et al.* (5), and isolated as the digitonide. Its radioactivity was determined as described elsewhere (6).

Results

In the first experiment the two rats described as eviscerated (Table I) were deprived of their gastrointestinal tracts, spleens, pancreases, and in addition their livers were excluded from the circulation. These rats converted acetate to cholesterol.

It was recently shown that the adrenal cortex is a site for sterol synthesis (6). Hence, in the second experiment, rats were not only eviscerated but also deprived of adrenal and ovarian tissues. Such preparations still retained the capacity to synthesize cholesterol from acetate (Table II, Rats 5, 6, and 7). The synthesis of cholesterol, therefore, is not restricted to a few tissues but is of reasonably widespread occurrence in the rat.

Bloch and Rittenberg were unable to demonstrate the *in vitro* conver-

TABLE I

Conversion of Injected C¹⁴-Labeled Acetate to Cholesterol by Normal and Eviscerated Rats

Rat No.	Weight	Treatment	Acetate Injected*		Time killed after 1st injection	Cholesterol isolated		
						Per cent injected C ¹⁴ recovered	Specific activity†	Total c.p.m. isolated
	gm.		mm	c.p.m.	hrs.			
N1	270	None	0.027	2.7×10^6	6	0.6	40	1.6×10^4
N2	290	"	0.027	2.7×10^6	6	0.4	28	1.1×10^4
H1	330	Eviscerated	0.026	2.6×10^6	6	0.2	13	5.2×10^3
H2	320	"	0.026	2.6×10^6	5.8	0.1	7	2.6×10^3

* The amounts recorded were injected in three equal portions during 6 hours. Its specific activity was 1.22×10^6 counts per minute per mg. of acetate.

† The cholesterol was isolated as the digitonide. The specific activity of the cholesterol was calculated by multiplying the counts per minute per mg. of the digitonide by 4.18, which is the value for the ratio of the molecular weight of cholesterol digitonide to cholesterol.

TABLE II

Conversion of Injected C¹⁴-Labeled Acetate to Cholesterol by Normal Rats and Rats Deprived of Various Organs

Rat No.	Weight	Treatment	Acetate injected (0.12 mm)		Duration	Specific activity of isolated cholesterol*
			C.p.m.	Specific activity		
	gm.			c.p.m. per mg.	hrs.	
1	202	None	7.9×10^6	8.0×10^6	6	83
2	208	"	6.9×10^6	7.0×10^6	6	155
3	194	"	5.6×10^6	5.7×10^6	5	62
4	196	Eviscerated†	5.7×10^6	5.8×10^6	5	47
5	194	" † ovariectomized; adrenalectomized	7.9×10^6	8.0×10^6	6	36
6	215	Eviscerated, ovariectomized; adrenalectomized	6.9×10^6	7.0×10^6	4.5	50
7	210	Eviscerated, ovariectomized; adrenalectomized	6.9×10^6	7.0×10^6	6	81
8	180	Ovariectomized; adrenalectomized	7.9×10^6	8.0×10^6	5	105

* See the foot-note, Table I. Total cholesterol was not isolated in these experiments.

† These rats were deprived of their gastrointestinal tracts, spleens, and pancreases, and their livers were deprived of circulation. See the text for the description of operative procedure.

sion of acetate to cholesterol in tissues other than the liver. They tested kidney, spleen, testes, and gastrointestinal tract, using deuterated acetate (3). We reinvestigated this *in vitro* reaction with the aid of the more sensitive indicator, C¹⁴. The results are shown in Table III.

TABLE III

In Vitro Conversion of C¹⁴-Labeled Acetate to Cholesterol by Isolated Tissues of Rat

Each flask contained 500 mg. of tissue and 5 cc. of Krebs-Henseleit bicarbonate buffer (pH 7.4) containing C¹⁴H₃C¹⁴OONa. Incubated 3 hours at 37.5°. The atmosphere in the flask was 95 per cent O₂-5 per cent CO₂.

Tissue	Acetate added to medium*	Weight of tissue per run	Cholesterol isolated	
			Per cent added C ¹⁴	Specific activity of cholesterol†
	c.p.m. × 10 ⁵	gm.		× 10 ²
Liver.....	2.6	1.0	0.67	10
“	2.6	1.0	0.83	11
Gut.....	2.6	1.0	0.34	5.1
“	2.6	1.2	0.60	8.8
Testis.....	3.1	1.0	0.29	4.8
“	3.1	1.0	0.24	4.3
Kidney.....	3.1	1.1	0.10	0.77
“	3.1	1.0	0.07	0.56
Adult skin.....	3.1	0.8	0.52	11
“ “	3.1	1.0	0.45	12
“ “	2.6	1.0	0.43	11
“ “	2.6	1.0	0.41	12
Baby skin.....	2.7	1.0	1.4	28
“ “	2.7	1.0	1.3	19
Adult brain.....	2.6	1.0	0	0
“ “	2.6	1.0	0	0
“ “ ‡.....	11	5.0	0	0
“ “	11	5.0	0	0
Baby “ ‡.....	2.7	1.1	4.2	24
“ “	2.7	1.0	3.5	21

* The specific activity of this acetate was 1.22 × 10⁶ counts per minute per mg.

† See the foot-note, Table I.

‡ The medium in which the brain slices were suspended contained 400 mg. per cent of glucose.

The most active tissues were found to be skin and liver of the adult and skin and brain of the day-old rat, as judged by both the percentage of the C¹⁴ added to the bath that was converted to cholesterol and the specific activities of the isolated cholesterol.

The demonstration that testis converts acetate to cholesterol *in vitro*

was not unexpected in view of its rôle in the synthesis of a steroidal hormone.

The high cholesterol content of skin prompted us to test that tissue. In order to obtain skin free of extraneous tissue and hair we used new born rats. This tissue was found to be active in synthesizing cholesterol. Adult rat skin from which the surface hair was removed was also tested and found active in converting acetate to cholesterol.

Kidney slices and small intestine also synthesized cholesterol at the expense of added acetate.

In 1940 Waelsch, Sperry, and Stoyanoff (7) injected rats of varying ages with heavy water and, on the basis of atom per cent excess of the unsaponifiable fraction, argued that the lipides deposited in the rat brain between the 15th and 19th day of life are synthesized in the brain itself. Table III shows that surviving brain preparations obtained from the day-old rat are quite active in converting acetate to cholesterol. This capacity to synthesize cholesterol is, however, lost with advancing age; slices prepared from *adult* rat brain completely failed to synthesize cholesterol from acetate.

The absence of measurable counts in the cholesterol fraction isolated from adult rat brain slices which had been incubated with C^{14} -labeled acetate establishes the validity of the procedure used here for isolating cholesterol free of contaminating radioactivity.

DISCUSSION

The results of the present investigation should serve to dispel the view that the liver is the only site in the body for the synthesis of cholesterol. But they should not be interpreted as minimizing the importance of this organ in cholesterol formation.

In our first study of extrahepatic cholesterol synthesis, we compared the conversion of acetate to cholesterol by the normal and eviscerated rat. A short term experiment, namely one of 6 hour duration, was chosen in order to avoid complications that occur late after hepatectomy. Skin, in view of its bulk and the extent of its activity, probably accounts, in large measure, for the cholesterol synthesis reported here for the eviscerated animal.

It is not possible, on the basis of either the percentage recovery of C^{14} in isolated cholesterol or the specific activities of this cholesterol, to compare the various tissues with respect to their rates of cholesterol turnover. The difficulties in obtaining, with tracers, an exact measure of turn-over rates from *in vitro* slice experiments have been discussed by Schachner *et al.* (8) and by Kalckar *et al.* (9). If it is assumed, however, that the rates of penetration of acetate to the site of cholesterol formation or the

rates of acetate production within the slice did not differ widely among the tissues studied here, it may be concluded that the tissues most concerned with cholesterol synthesis in the adult animal body are skin and liver.

SUMMARY

1. Functionally hepatectomized rats can convert acetate to cholesterol.
2. In addition to liver and adrenal, the following tissues of the adult rat are capable of converting acetate to cholesterol: kidney, testis, small intestine, and skin.
3. The brain and skin of the new born rat also synthesized cholesterol.
4. Surviving brain slices of adult rats completely failed to convert acetate to cholesterol.
5. Hepatic and extrahepatic cholesterol synthesis is discussed.

BIBLIOGRAPHY

1. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **159**, 45 (1945).
2. Anker, H. S., *J. Biol. Chem.*, **176**, 1337 (1948).
3. Bloch, K., Borek, E., and Rittenberg, D., *J. Biol. Chem.*, **162**, 441 (1946).
4. Griffith, J. Q., Jr., and Farris, E. J., *The rat in laboratory investigation*, Philadelphia, 389, 393 (1942).
5. Sperry, W. M., Waelsch, H., and Stoyanoff, V. A., *J. Biol. Chem.*, **135**, 281 (1940).
6. Srere, P. A., Chaikoff, I. L., and Dauben, W. G., *J. Biol. Chem.*, **176**, 829 (1948).
7. Waelsch, H., Sperry, W. M., and Stoyanoff, V. A., *J. Biol. Chem.*, **135**, 297 (1940).
8. Schachner, H., Fries, B. A., and Chaikoff, I. L., *J. Biol. Chem.*, **146**, 95 (1942).
9. Kalckar, H. M., Dehlinger, J., and Mehler, A., *J. Biol. Chem.*, **154**, 275 (1944).

PLUTONIUM AND YTTRIUM CONTENT OF THE BLOOD, LIVER, AND SKELETON OF THE RAT AT DIFFERENT TIMES AFTER INTRAVENOUS ADMINISTRATION*

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In the course of investigations devoted to the development of procedures for removing radioelements from the body, it became necessary to determine the immediate rates with which soluble tetravalent and trivalent radioelements leave the blood and become incorporated in the various tissues. Plutonium (Pu^{239}) and yttrium (Y^{91}) were chosen for this study because their cations are fairly representative of tetravalent and trivalent species, respectively, and because much is already known concerning their metabolism.

EXPERIMENTAL

Eighteen female Sprague-Dawley rats, weighing between 236 and 254 gm., received an injection of a 1 per cent sodium citrate solution containing both Pu^{239} and Y^{91} into one of the lateral tail veins. At the same time equivalent amounts of solution were placed in several volumetric flasks ("dummy" injections). The solution of $\text{Pu} + \text{Y}$ was prepared in such a way that no colloidal states were present at the time of injection.¹ This was done by diluting a small volume of an acid solution of $\text{Pu} + \text{Y}$ with an excess of 2 per cent citric acid and then carefully adjusting the pH to 6.5 with NaOH. The resulting solution was diluted so that it was isotonic with blood and contained the equivalent of 1 per cent citric acid. The metabolism of Pu or Y at tracer levels is not altered significantly by forming a complex with citrate (1-3). Each animal received 0.4 cc. of the solution, which contained 24.3 γ of Pu (+4), giving 1.7×10^6 counts per minute under our counting conditions, and 0.3 $\mu\text{c.}$ of Y (+3), giving 4×10^3 counts per minute.

The animals were sacrificed in pairs at 5, 15, 30, and 60 minutes, and at 2, 6, 12, 24, and 48 hours. Blood, bone, and liver were selected for analysis since previous experiments had shown that the latter two tissues contain most of the injected Pu and Y (1, 3, 4). A measured volume of blood was

* We wish to acknowledge the technical assistance of Rosie M. Hunter.

¹ Ultrafiltration studies have shown that all of the Pu and Y in these solutions is completely diffusible.

obtained by cardiac puncture, after which the animal was killed with nembutal. The right femur was removed and weighed (weight range, 0.90 to 0.99 gm.) and the liver was removed, gently blotted free of adhering blood, and weighed (weight range, 6.6 to 7.8 gm.). From the femur and the blood sample, respectively, the Pu content and the Y content of the entire skeleton and the blood were estimated.²

The liver and the samples of blood and bone were digested with concentrated nitric acid and evaporated to near dryness. This ashing procedure was repeated, usually three to four times, until only a white ash remained. The ash was dissolved in concentrated nitric acid and diluted to 100 cc. in a volumetric flask. Measured aliquots were taken for counting. For the determination of Y, the sample was deposited directly in a porcelain dish and slowly dried under an infra-red bulb; for the Pu determination it was deposited on a platinum dish, dried by infra-red light, and then flamed.

Inasmuch as Pu is an α emitter and Y a β emitter, it was possible to count 1 isotope in the presence of the other. Sufficient activities were present so that chemical separation was not required. The radioactivity of each sample was compared to a sample prepared in identical manner from one of the dummy injection solutions to which had been added equivalent ashed tissue from an animal which had had no injection; in this way positive or negative corrections due to self-absorption or geometry changes were largely eliminated (6). The α radiations were measured under 50 per cent geometry in an ionization chamber adjusted to record pulses due only to α -particle ionization. The β -particles were counted through a 13.5 mg. per sq. cm. aluminum absorber with a thin mica window Geiger-Müller tube. The aluminum absorber served to exclude the radiations contributed by the plutonium. In previous experiments with Pu and Y these procedures have given consistent recoveries of about 95 ± 5 per cent of the injected dose.

Results

The changes with time in the levels of Pu and Y in the blood, skeleton, and liver of the rat are shown in Figs. 1 and 2. 5 minutes after intravenous injection about 50 per cent of the Pu and 80 per cent of the Y had disappeared from the blood, and an appreciable fraction of each already was deposited in the liver and skeleton. After this initial sharp drop Pu disappeared more slowly from the blood; 12 hours after administration about

² The amount of Pu and Y in the skeleton was taken to be 20 times that contained in a single femur. This is close to the factor of 24 given in Donaldson's tables (5) for the ratio of the rat's skeletal weight to the weight of a single femur. The total blood volume was estimated to be 6.06 per cent of the body weight in gm. (5).

20 per cent of the injected dose was still being circulated, while the Y content of the blood had decreased to 0.7 per cent. These data are in good agreement with the observations of Painter *et al.* (7) on the dog, and Van

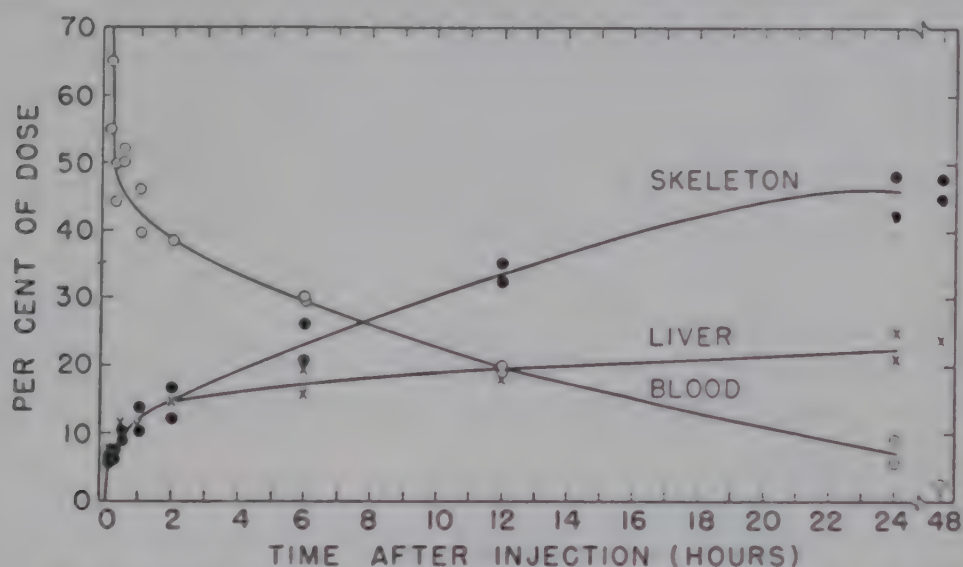


FIG. 1. Pu²³⁹ content of blood, liver, and skeleton as a function of time after intravenous injection. In cases in which only one point is shown for a time interval, the values for the two rats were almost identical.

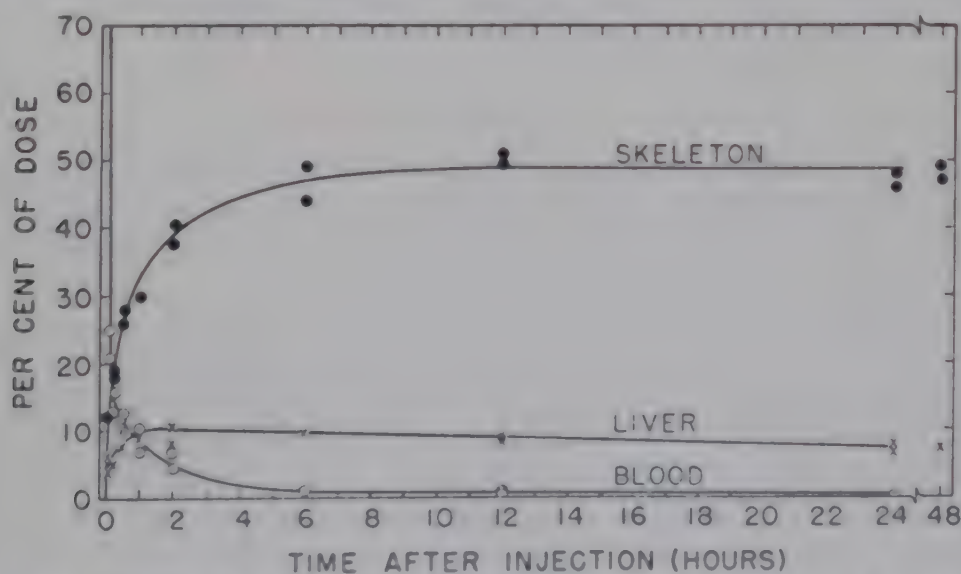


FIG. 2. Y⁹¹ content of blood, liver, and skeleton as a function of time after intravenous injection. In cases in which only one point is shown for a time interval, the values for the two rats were almost identical.

Middlesworth (8) on the rat. The latter investigator found that 30 minutes after intravenous administration of Pu (+4) the blood of rats contained about 50 per cent of the injected dose, whereas only 10 per cent remained when Pu (+6) was given.³

³ Pu (+6), *i.e.* PuO₂⁺⁺ can be considered to be a member of the alkaline earth group of elements.

The Pu content of the skeleton increased regularly until a maximum value of about 45 per cent of the injected dose was reached at 24 hours; in 6 hours the Y concentration was near its peak of 45 per cent. The uptake of Pu and Y by the skeleton is closely paralleled, as is the case with the alkaline earths (9), by the rate of disappearance of these elements from the blood stream. Only 2 hours are required for the level of intravenously injected alkaline earths (Ca, Sr, Ra) to reach a maximum in bone (9). It has been shown that within a month the skeleton contains more than 80 per cent of the Pu and Y still present in the body (1, 8).

After the initial rapid increase of the Pu and Y values in the liver, the level of Pu continued to rise at a diminished rate until it reached 24 per cent of the injected dose at 24 hours. However, the Y level decreased very slowly after the 2nd hour, at which time the maximum value of close to 10 per cent was attained.

The amounts of Pu and Y eliminated from the rat under conditions similar to those described in this paper have been measured. It has been found that during the first 24 hours following injection the rat excretes about 3 per cent of the Pu and 25 per cent of the Y (1, 3, 10). Almost immediately thereafter the rate of excretion drops to such low levels that the body content of Pu and Y remains nearly constant.

DISCUSSION

Consideration of the solution chemistry of radioelements aids in the interpretation of the results described above. When a cationic, carrier-free radioelement is injected into the blood stream, its subsequent solution chemistry becomes very complex. It can react in one or more of four principal ways: (a) combine with anions such as phosphate to form salts of low solubility; (b) combine with proteins; (c) polymerize as a result of repeated hydroxylations (11); or (d) form soluble complex ions by combining with anions such as citrate. Reaction (a), (b), or (c) generally results in the formation of colloidal or colloidal-like particles of low diffusibility (12). Those elements which remain simple ions or form the complexes mentioned in (d) display high diffusibility.

The rate and extent of the polymerization of a cationic radioelement increase with increasing concentration and decreasing basicity. Qualitatively, the percentage of the soluble salt of an injected carrier-free element that leaves the circulation within the first 5 or 10 minutes appears to increase with a decreasing tendency of the radioelement to polymerize at the pH of blood. Presumably, these rapidly disappearing fractions are essentially simple diffusible ions which have not yet polymerized or combined with the plasma proteins or, in other words, become colloidal. The propensity to polymerize can be roughly gaged by the pH at which the

hydroxide of a dilute solution of a simple salt of the element in question will precipitate. For example, tetravalent plutonium salts in simple dilute solution precipitate at a pH of 2 to 3 (11) and therefore leave the blood more slowly than do those of yttrium, which do not precipitate until a pH of about 7 is reached (13). Thus, the largest fraction (about 80 per cent) of the Y, which disappears from the blood within 5 minutes, is probably in the form of soluble ions. This is borne out by the fact that only a small amount was deposited in the liver.

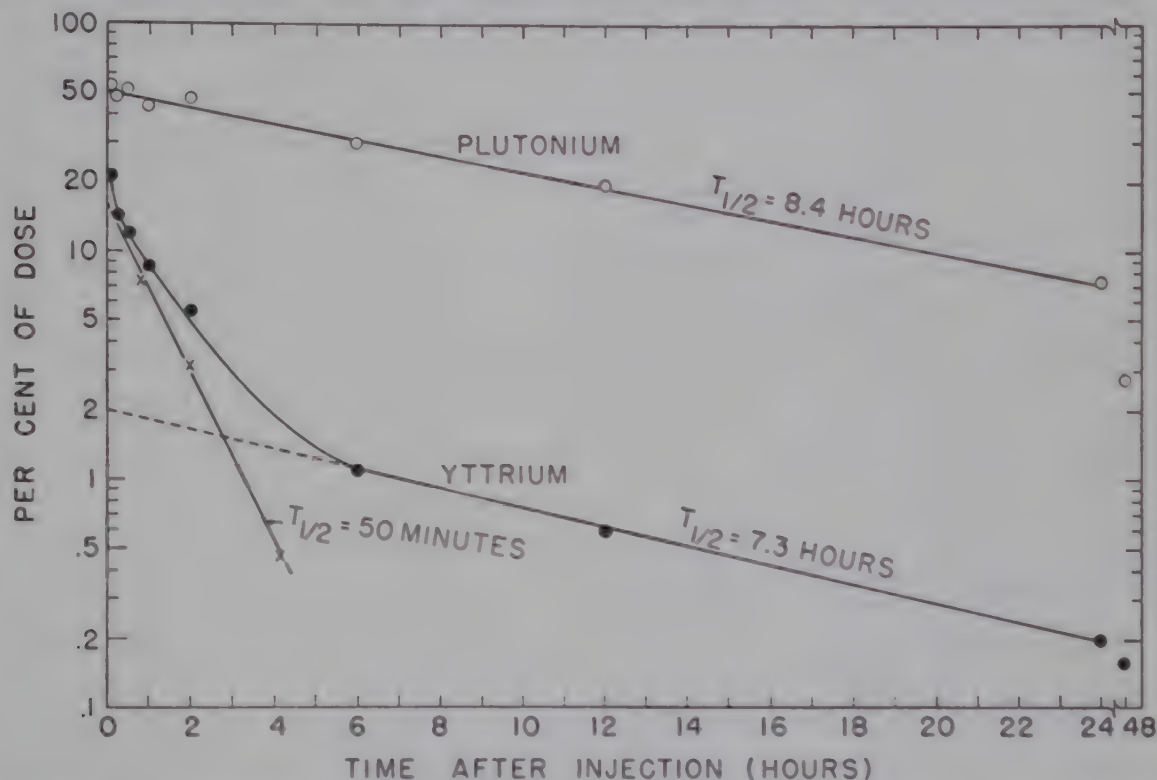


FIG. 3. Disappearance of Pu^{239} and Y^{91} from the circulating blood after intravenous injection. The circles represent the average values obtained from two rats at each time interval. The straight line drawn through the \times points was obtained by subtracting the extrapolated dotted line from the curved portion of the yttrium curve.

Dobson and his coworkers (14) have shown that the rate of removal of colloids from the circulating blood is related to particle size. Apparently, most of the large colloids are cleared from the blood in a single passage through the liver ($t_{\frac{1}{2}} = \sim 1$ minute), while smaller particles are withdrawn more slowly ($t_{\frac{1}{2}} = 30$ to 80 minutes). In the present experiment 50 per cent of the injected Pu and 2 per cent of the injected yttrium disappeared very slowly, $t_{\frac{1}{2}} = 8$ and 7 hours, respectively (Fig. 3). The long half life in the blood stream of these fractions can be explained, at least in part, on the basis of the formation of very small colloids. The maximum concentration of Pu in the blood was less than 10^{-6} M, and diffusion studies made in isotonic inorganic media indicate that at this concentration Pu exists in the form of colloidal-like particles with a diameter of 40 to 50

A.⁴ The 18 per cent of the Y that left the blood with a half time of 50 minutes probably falls in the same particle-size category as the zirconium and yttrium colloids ($t_{1/2} = 30$ to 80 minutes) described by Dobson *et al.* (14).

The concept that the rate of passage of substances through the capillary endothelium *in vivo* is directly related to the diffusion rate *in vitro* was proposed by Cohnheim in 1898 (15). Schulemann (16) demonstrated this

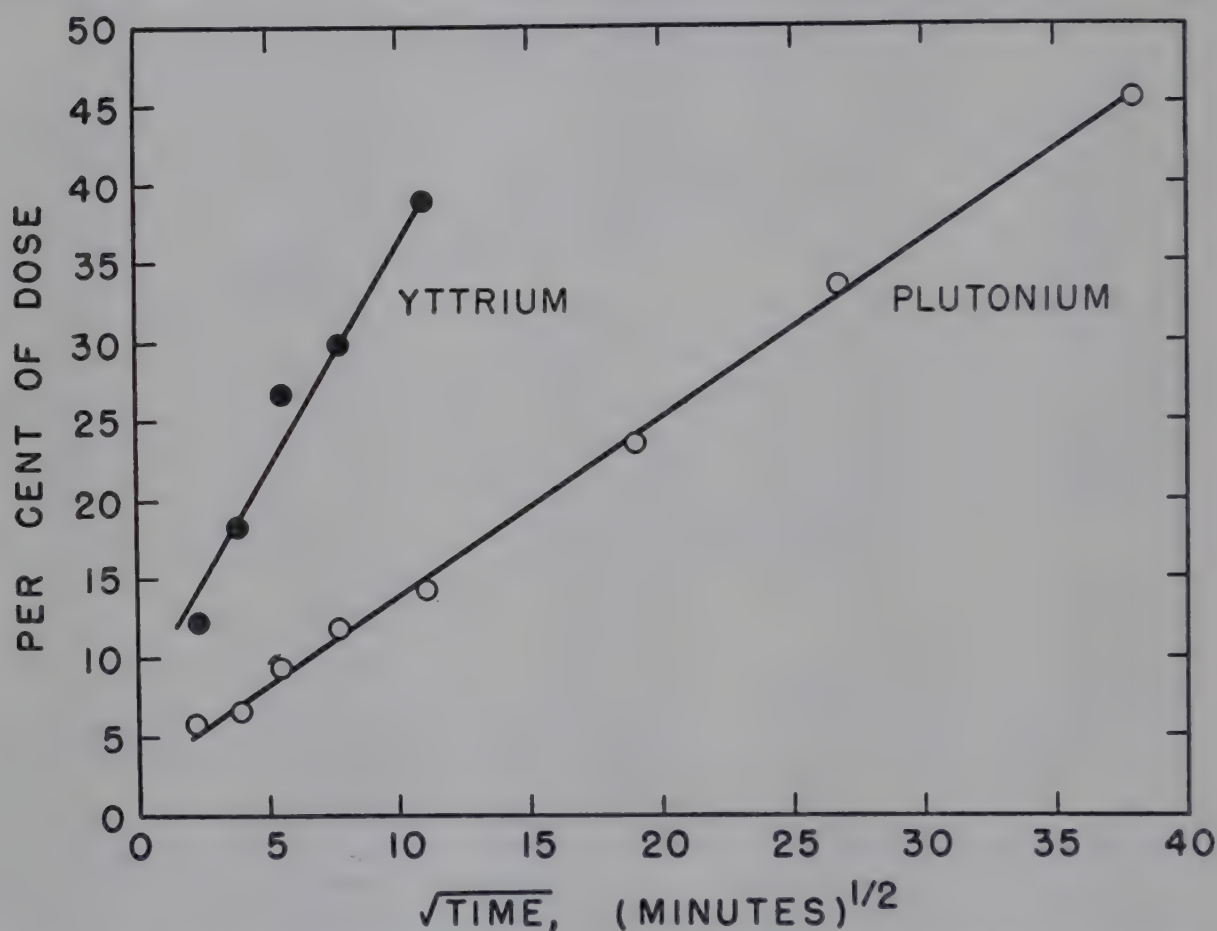


FIG. 4. Rate of uptake of Pu^{239} and Y^{91} by the skeleton according to the parabolic diffusion law. The points plotted represent the average value obtained from two rats at each time interval.

relationship with organic dyes. After subcutaneous injections into mice or rabbits, the dyes were distributed in the organism and in particular cells at the same relative rates as they diffused through gelatin. Clark (17) showed that the rate of absorption of inorganic salts and glucose through the peritoneum varied according to the rate of diffusion in water or gelatin.

If diffusion rates govern the uptake of substances in the body, then, in accordance with the simple theory of diffusion in solids, the amount of

⁴ Schubert, J., and Conn, E. E., unpublished work.

material absorbed should be a linear function of the square root of time. This can be expressed in terms of the parabolic diffusion law (18) as

$$y = k \sqrt{t}$$

in which y = the per cent of uptake of the element, t = time, and k = a constant that includes \sqrt{D} , D being the diffusion coefficient.

Some form of this equation has been applied to many phenomena, including the rates of anion exchange in ion exchange resins (19), the adsorption of phosphates by powdered bone (20, 21), the disappearance of injected dyes from the blood stream (22), the occlusion of solutes by zeolitic materials (18), and the capillary movement of water in soil (23). When the equation is applied to our data for the skeletal uptake of plutonium (Fig. 4), a linear relationship is obtained up to the plateau value. In the case of yttrium this is true up to 80 per cent of the plateau value. The fact that extensions of the straight lines do not go through zero indicates that during the first few minutes a small fraction of each radioelement was taken up by very rapid processes other than diffusion. The rate constant, k , determined from Fig. 4, is 1.3 for Pu and 3.6 for Y; hence it is estimated that the diffusion velocity of Y into bone is 7 times greater than that of plutonium.

Combining our results with those of Norris and Kisielecki (9), we find the following order for the rate of uptake by bone: Sr (+2) > Y (+3) > Pu (+4). This relationship also holds for the relative proportion of the radioelement which disappears from the blood stream within the first 5 or 10 minutes. Inasmuch as the diffusion coefficient of a cation varies inversely with its valence, these data furnish additional suggestive evidence for a diffusion-controlled mechanism.

SUMMARY

1. 5 minutes after intravenous injection 50 per cent of the Pu²³⁹ (+4) and 80 per cent of the Y⁹¹ (+3) had left the blood stream. Within 6 hours almost all of the Y had disappeared from the blood; 48 hours were required for the removal of most of the Pu.

2. The maximum uptake of both Pu and Y by the skeleton was approximately 45 per cent of the injected dose. This was reached at 24 hours in the case of Pu and at 6 hours in the case of yttrium.

3. By the 24th hour the Pu content of the liver reached a maximum of 24 per cent of the injected dose; the Y content reached a maximum of 10 per cent by the 2nd hour and subsequently decreased at a very slow rate.

4. Interpretation of these data on the basis of a diffusion-controlled mechanism is discussed.

BIBLIOGRAPHY

1. Carritt, J., Fryxell, R., Kleinschmidt, J., Kleinschmidt, R., Langham, W., San Pietro, A., Schaffer, R., and Schnap, B., *J. Biol. Chem.*, **171**, 273 (1947).
2. Copp, D. H., Axelrod, D. J., and Hamilton, J. G., *Am. J. Roentgenol.*, **58**, 10 (1947).
3. Schubert, J., *J. Lab. and Clin. Med.*, **34**, 313 (1949).
4. Scott, K. G., Axelrod, D. J., Fisher, H., Crowley, J. F., and Hamilton, J. G., *J. Biol. Chem.*, **176**, 283 (1948).
5. Donaldson, H. H., *Memoirs of The Wistar Institute of Anatomy and Biology*, Philadelphia, 2nd edition (1924).
6. Johnston, F., and Willard, J. E., *Science*, **109**, 11 (1949).
7. Painter, E., Russell, E., Prosser, C. L., Swift, M. N., Kisieleski, W., and Sacher, G., AECD-2042, Technical information division, Oak Ridge directed operations, U. S. Atomic Energy Commission.
8. Van Middlesworth, L., MDCC-1022, Technical information division, Oak Ridge directed operations, U. S. Atomic Energy Commission.
9. Norris, W. P., and Kisieleski, W. E., *Cold Spring Harbor Symposia Quant. Biol.*, **13**, 164 (1948).
10. Schubert, J., *Science*, **105**, 389 (1947).
11. Kraus, K. A., MDCC-814, Technical information division, Oak Ridge directed operations, U. S. Atomic Energy Commission.
12. Schubert, J., and Conn, E. E., *Nucleonics*, **4**, 2 (1949).
13. Britton, H. T. S., *Hydrogen ions*, New York, 79 (1943).
14. Dobson, E. L., Gofman, J. W., Jones, H. B., Kelly, L. S., and Walker, L. A., *J. Lab. and Clin. Med.*, **34**, 305 (1949).
15. Cohnheim, *Habilitationsschrift*, Munchen (1898); cited by Clark in (17).
16. Schulemann, W., *Biochem. Z.*, **80**, 1 (1917); cited by Krogh, A., in *The anatomy and physiology of capillaries*, New Haven, revised edition, 278 (1929).
17. Clark, A. J., *J. Pharmacol. and Exp. Therap.*, **16**, 415 (1921).
18. Barrer, R. M., and Ibbitson, D. A., *Tr. Faraday Soc.*, **40**, 206 (1944).
19. Kunin, R., and Myers, R. J., *J. Phys. and Colloid. Chem.*, **51**, 1111 (1947).
20. Johansson, E. G., Falkenheim, M., and Hodge, H. C., *J. Biol. Chem.*, **159**, 129 (1945).
21. Hodge, H. C., and Falkenheim, M., *J. Biol. Chem.*, **160**, 637 (1945).
22. King, B. G., Cole, K. S., and Oppenheimer, E. T., *Am. J. Physiol.*, **138**, 636 (1943).
23. Kirkham, D., and Feng, C. L., *Soil Sc.*, **67**, 29 (1949).

ON THE EVIDENCE FOR A TERNARY COMPLEX OF CATALASE HYDROGEN PEROXIDE WITH ALCOHOLS*

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The decrease of the velocity constant for the reaction of the primary catalase-hydrogen peroxide complex (Complex I) with alcohols at higher concentrations of the latter has suggested that the enzyme-substrate complex combines with the acceptor molecule (AH_2) to form a ternary complex (1, 2) thus:



At high acceptor concentrations, the rate-determining step would be the first order decomposition of a ternary complex.



The velocity of disappearance of the ternary complex would be independent of the acceptor concentration.

$$-\frac{d\begin{array}{c} \text{AH}_2 \\ | \\ \text{Cat}(\text{OH})_3(\text{OOH})-\text{I} \end{array}}{dt} = k_5 \begin{array}{c} \text{AH}_2 \\ | \\ \text{Cat}(\text{OH})_3(\text{OOH})-\text{I} \end{array} \quad (3)$$

Under these conditions k_4 of equation (1) would no longer be constant but would apparently decrease with increasing acceptor concentration.

Previous studies (1) clearly showed that k_4 decreased markedly above about 10 mM acceptor concentration. However, this effect was not observed when alkyl hydrogen peroxides were used as substrates (3). Furthermore, the reaction of the catalase-hydrogen peroxide complex with hydrogen peroxide does not show this effect, at least up to 0.3 M hydrogen peroxide (4).

Recently, LuValle and Goddard (5) have generalized the concept of the ternary complex of enzyme, substrate, and acceptor and have assumed that such a complex exists in catalase reactions.

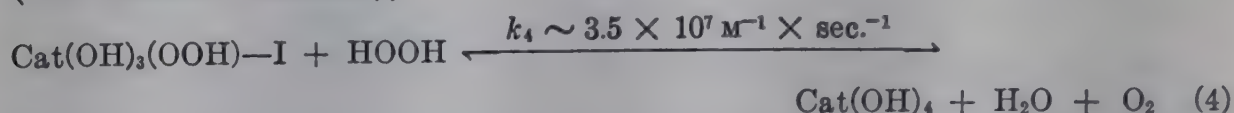
For these reasons, it is appropriate to present a more critical study of the evidence for a ternary complex in the reaction of catalase hydrogen peroxide and alcohols.

* This is paper 15 of a series on catalases and peroxides.

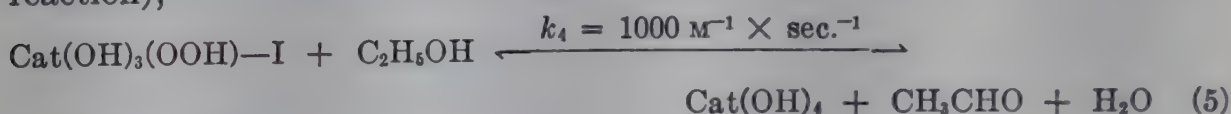
Methods

The catalase preparations have been described previously (1), as has the rapid spectrophotometric method for the measurement of the reaction of Complex I with alcohols (1).

Since the velocity of the reaction of Complex I with hydrogen peroxide (the catalatic reaction),



is much more rapid than the reaction with alcohols (the peroxidatic reaction),



reaction (4) would be expected to be very nearly complete before reaction (5) began. And under these conditions the two reactions could be measured separately; reaction (5) would occur essentially in the absence of free hydrogen peroxide in the solution. Thus there is no turnover of catalase in equation (5); only 1 molecule of alcohol is oxidized per enzyme molecule.

The velocity of the reaction of Complex I with alcohols can be measured by the rate of disappearance of Complex I in the presence of a known excess of alcohol, $[\text{C}_2\text{H}_5\text{OH}]_0$. On the assumption that turnover of catalase does not occur, k_4 is computed from the half time of the reaction

$$k_4 = \frac{0.693}{t_{\frac{1}{2}}[\text{C}_2\text{H}_5\text{OH}]_0} \quad (6)$$

The catalase and hydrogen peroxide concentrations are not involved in this method of determining k_4 . The spontaneous breakdown of Complex I in the absence of added alcohol may be considerable and, in this case, the velocity constant for this reaction, k_3 , is subtracted from $0.693/t_{\frac{1}{2}}$.

$$k_4 = \left(\frac{0.693}{t_{\frac{1}{2}}} - k_3 \right) \frac{1}{[\text{C}_2\text{H}_5\text{OH}]_0} \quad (7)$$

Critique of Method—It is useful to make a rough calculation of the actual time separation of reactions (4) and (5) for typical experimental conditions for these methods. First, the time, t , required by reaction (4) to reduce the hydrogen peroxide concentration from an initial value, $[\text{H}_2\text{O}_2]_0$, to any value, $[\text{H}_2\text{O}_2]$, is calculated from the equation,

$$t = \frac{2.3}{ek'_1} \log \frac{[\text{H}_2\text{O}_2]_0}{[\text{H}_2\text{O}_2]} \quad (8)$$

where e is the molar catalase concentration and $k'_1 = 3.5 \times 10^7 \text{ M}^{-1} \times \text{sec.}^{-1}$.

If the value of $[H_2O_2]$ is so large that t of equation (8) $\approx t_1$ of equation (7), the hydrogen peroxide may interfere with the reaction with alcohol. If, during the reaction of equation (5), the hydrogen peroxide concentration is large compared to the dissociation constant of Complex I, then hydrogen peroxide will combine with free catalase and will sustain the concentration of Complex I, and turnover of catalase will occur, violating the assumption made in writing equation (6). Thus the spectrophotometrically measured value of t_1 will be larger than if the hydrogen peroxide concentration were negligible. To illustrate this error, the time, t , required for the hydrogen peroxide concentration to fall from its initial value to a value equal to the dissociation constant of Complex I is calculated as follows:

The dissociation constant of Complex I is approximately equal to the ratio of its rate of decomposition, $k_4[C_2H_5OH]_0$, to the velocity constant for its formation, k_1 ,

$$K_m = \frac{k_4[C_2H_5OH]_0}{k_1} \quad (9)$$

Thus, from equation (8),

$$t = \frac{2.3}{ek_1} \log \frac{[H_2O_2]_0}{K_m} = \frac{2.3}{ek_1} \log \frac{[H_2O_2]_0 k_1}{[C_2H_5OH]_0 k_4} \quad (10)$$

Equation (10) clearly shows that the enzyme concentration is much more important in determining t than are the peroxide or alcohol concentrations. Fig. 1 illustrates the hyperbolic relation between t and e for equal initial peroxide and alcohol concentrations. With concentrated catalase ($> 2 \mu M$), t is small (~ 0.1 second). With dilute alcohol (t_1 of equation (6) greater than 1 second), the assumptions on the time separation of reactions (4) and (5) are therefore justified: the disappearance of peroxide (the catalatic reaction) is substantially complete before the reaction with alcohol begins (the peroxidatic reaction). And the bulk of the previous data was obtained under these conditions.

With dilute catalase ($< 1 \mu M$), the values of t increase very rapidly and encroach upon the usual time range for t_1 . Although no quantitative relation between the value of t and the error of k_4 for a measured value of t_1 can be given at present, an increase of k_4 caused by an increase of enzyme concentration furnishes strong evidence that such an error may exist. A preliminary test of this type was made previously (Chance (1), Fig. 20) and showed no measurable error at that alcohol concentration (4 mM). This paper gives a more critical study of this error.

Relation between k_4 and Ethanol Concentration for Two Values of Catalase Concentration—This experiment (see Chance (1), Fig. 13) has been repeated with more dilute catalase (0.54 compared with $1.4 \mu M$) and with a

larger initial peroxide concentration. The data from both experiments are plotted in Fig. 2. First, it may be seen that the values of k_4 determined at the two catalase concentrations are in good accord ($k_4 = 1000 \text{ M}^{-1} \times \text{sec.}^{-1}$) at low alcohol concentrations at which t_1 is large. Second, it is clearly shown that at small values of t_1 , k_4 decreases, and begins to decrease at a smaller alcohol concentration with the more dilute enzyme. Curve A breaks at about 4 mM of ethanol, while Curve B breaks at about

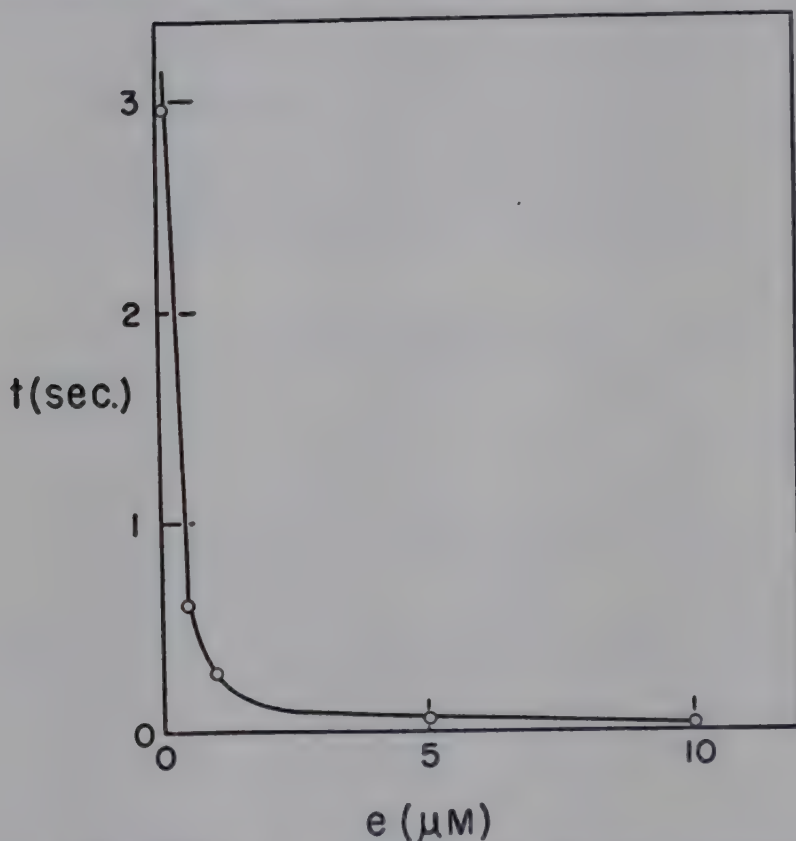


FIG. 1. A mathematical relation between catalase concentration and the time separation between the catalytic and peroxidatic reactions; a graph of equation (10) for $[\text{H}_2\text{O}_2]_0/[\text{C}_2\text{H}_5\text{OH}]_0 = 1$ and for $k_4 = 10^3$ and $k_1 = 3.5 \times 10^7 \text{ M}^{-1} \times \text{sec.}^{-1}$.

0.4 mM of ethanol. This effect is in accordance with the computations above.

Relation between k_4 and Catalase Concentration—The results of a more direct experiment are given in Fig. 3 where k_4 is measured at three enzyme concentrations and at a low and high ethanol concentration. At the low alcohol concentration, k_4 ($= 1060 \text{ M}^{-1} \times \text{sec.}^{-1}$) is independent of the enzyme concentration and the values of k_4 truly represent reaction (5); i.e., $t \ll t_1$. At the higher alcohol concentration, the value of k_4 is much smaller and increases with the enzyme concentration, $t \approx t_1$.

Thus there is qualitative agreement between the theory and experiment. We are, at present, unprepared to explain the apparently linear relation between k_4 and catalase concentration at high alcohol concentrations as shown in Fig. 3. On the basis of the simple theory presented here, we

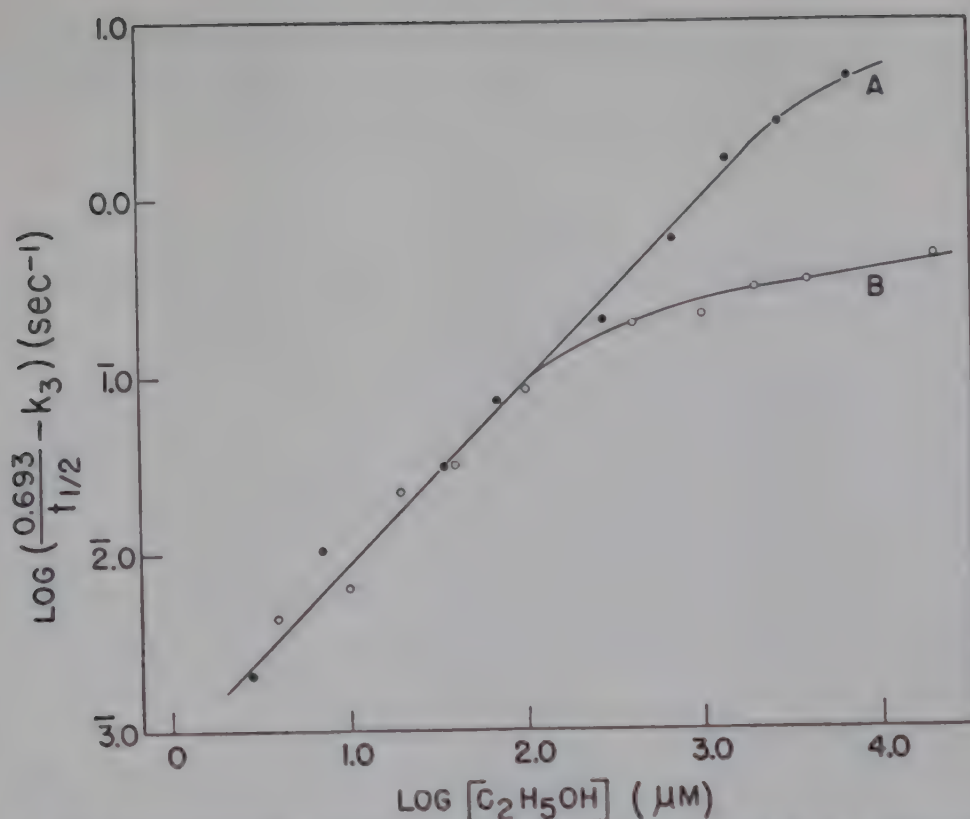


FIG. 2. The experimental relationship between the velocity of disappearance of Complex I and the concentration of ethanol for two values of catalase concentration. The value of k_4 is $1000 \text{ M}^{-1} \times \text{sec}^{-1}$. Curve A is from Chance (1), Fig. 13; $1.36 \mu\text{M}$ of horse blood catalase, $15 \mu\text{M}$ of H_2O_2 (Experiment 59); Curve B, 0.54 M of horse blood catalase, $200 \mu\text{M}$ of H_2O_2 (Experiment 373); pH 7.0, 0.01 M phosphate.

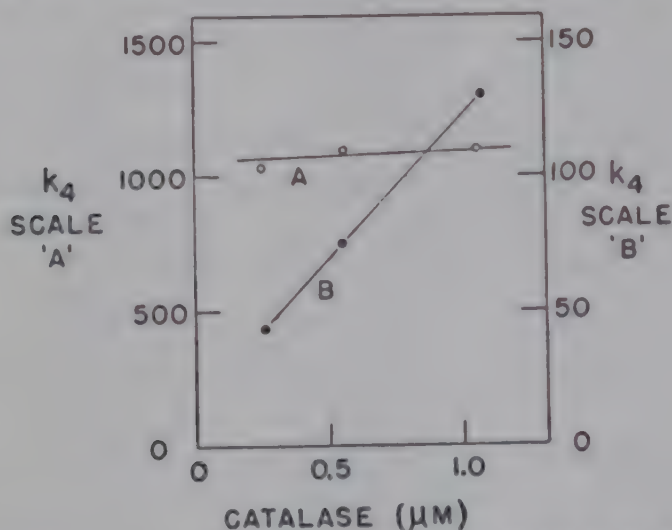


FIG. 3. Illustrating in Curve A the independence of k_4 and catalase concentration in the presence of dilute ethanol (0.4 mM) ($k_4 = 1060 \text{ M}^{-1} \times \text{sec}^{-1}$), and in Curve B, the dependence of k_4 upon catalase concentration in concentrated ethanol (40 mM); $100 \mu\text{M}$ of H_2O_2 , pH 7.0, 0.01 M phosphate (Experiment 373j).

would have expected a more rapid increase of k_4 . The mathematical approach presented here gives only qualitative agreement with the experimental data; a more comprehensive analysis would shed light on the detailed mechanisms of these reactions.

It is now apparent that the value of alcohol concentration and the range of enzyme concentration were insufficient to illustrate this effect clearly in the previous test (Chance (1), Fig. 20).

SUMMARY

This paper presents a mathematical analysis and an experimental verification of an error which may arise in the determination of the velocity constant (k_4) for the reaction of catalase hydrogen peroxide with alcohol due to the choice of improper experimental conditions. At $1.5\ \mu\text{M}$ or higher catalase concentrations, k_4 , as measured by this method, is constant up to 4 mM of alcohol. At $0.5\ \mu\text{M}$ catalase, k_4 is constant up to 0.4 mM of alcohol. At higher alcohol concentrations k_4 is shown to decrease and this decrease is attributed to the fact that the catalytic reaction is not complete before the peroxidatic reaction begins. This decrease of k_4 is no longer considered to be caused by the formation of a ternary complex of catalase, hydrogen peroxide, and ethanol as in a previous paper (1).

If a hypothetical ternary complex of enzyme, substrate, and acceptor exists, its life time is too short to be revealed by these methods. The suitability of a test for such a ternary complex is indicated by the maximum turnover number ($k_4[\text{acceptor}]$) that can be measured. Experiments with catalase hydrogen peroxide and alcohol give turnover numbers up to $4\ \text{sec}^{-1}$, with catalase methyl hydrogen peroxide and alcohol $80\ \text{sec}^{-1}$ (6), and with catalase hydrogen peroxide and hydrogen peroxide (the catalytic reaction) up to $10^7\ \text{sec}^{-1}$ ($3.5 \times 10^7 \times 0.3\ \text{M}$) (4). Since the values of k_4 are constant at these values of turnover number, the life time of such a ternary complex in peroxidatic activity is less than $1/80$ second and, in catalytic activity, less than 10^{-7} second. It cannot be said, at present, whether such short life times are probable or improbable. Therefore, the general theory of the ternary complex is not furthered by these data.

The conditions for the spectrophotometric determination of the velocity constant for the reaction of catalase hydrogen peroxide with alcohols have been defined. The decrease of this reaction-velocity constant at low catalase and high alcohol concentrations is attributed to the reaction mechanisms and not to the formation of a catalase-hydrogen peroxide-alcohol complex.

BIBLIOGRAPHY

1. Chance, B., *Acta chem. Scand.*, **1**, 236 (1947).
2. Chance, B., *Science*, **109**, 204 (1949).
3. Chance, B., *J. Biol. Chem.*, **179**, 1341 (1949).
4. Bonnichsen, R. K., Chance, B., and Theorell, H., *Acta chem. Scand.*, **1**, 685 (1947).
5. LuValle, J. E., and Goddard, D. R., *Quart. Rev. Biol.*, **23**, 197 (1948).
6. Chance, B., *J. Biol. Chem.*, **182**, 649 (1950).

ON THE REACTION OF CATALASE PEROXIDES WITH ACCEPTORS*

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The first study of the specificity of catalase for various alcohols was made by Keilin and Hartree (1) in their second paper on coupled oxidations. Later Chance (2) measured the velocity constants for the reactions of the catalase-hydrogen peroxide and catalase-alkyl hydrogen peroxide complexes with various alcohols and reported that formate and methylene glycol react similarly (3). Heppel and Porterfield (4) have obtained evidence, based upon coupled oxidations, that nitrite is oxidized to nitrate by catalase and hydrogen peroxide.

The velocity constants for the reactions of nitrite, methylene glycol, and formate with the catalase-peroxide complexes are given in this paper and may be compared with the values for methanol and ethanol obtained previously.

The reaction of catalase methyl hydrogen peroxide with ethanol has been measured at high ethanol concentrations to determine whether the formation and breakdown of a ternary complex of enzyme, substrate, and acceptor¹ limit the catalase turnover number.

The reactions of catalase hydrogen peroxide with methylene glycol and catalase methyl hydrogen peroxide with nitrite do not proceed rapidly at higher acceptor concentrations, and a study of the possible causes of these effects is presented.

The effect of ascorbic acid upon the rate of breakdown of catalase hydrogen peroxide is discussed.

Method

The catalase and methyl hydrogen peroxide preparations have been discussed previously (5).

The rapid spectrophotometric method is used for measuring the reaction kinetics of the primary catalase-peroxide complexes and the velocity con-

* This is Paper 16 of a series on catalases and peroxides.

¹ The definition of acceptor given previously (3) is inaccurate and is amended to read: The acceptor is defined as the molecule that is oxidized by the enzyme-substrate complex.

stants (k_4) for the reactions with the various acceptors (a_0) are computed as follows: For catalase hydrogen peroxide,

$$k_4 a_0 = \left[\frac{0.693}{t_{\frac{1}{2}}} - k_3 \right] \quad (1)$$

where $t_{\frac{1}{2}}$ is the half time for the decomposition of the catalase-hydrogen peroxide complex and k_3 is the velocity constant for the spontaneous de-

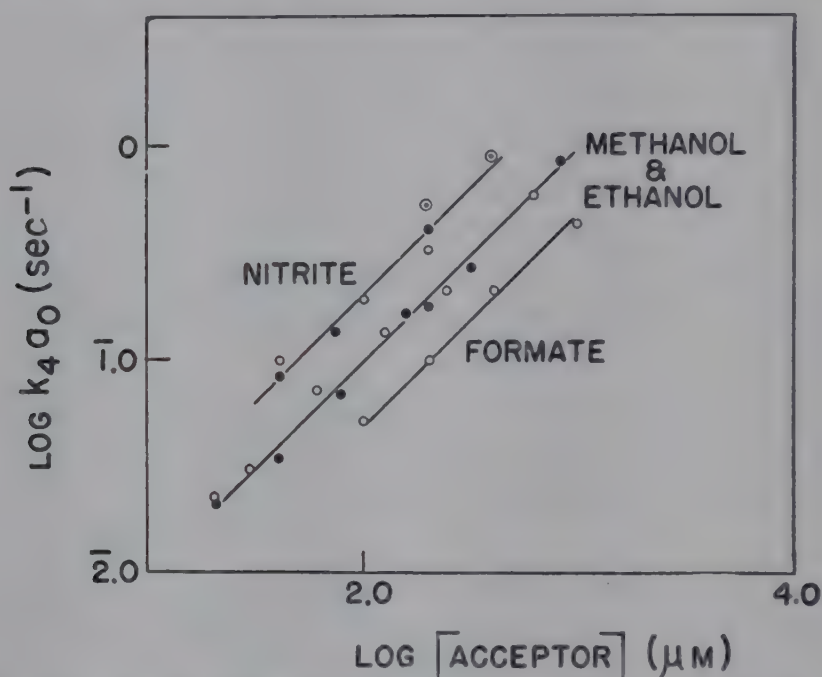


FIG. 1. The relation between reaction velocity and concentration for the reaction of nitrite and formate with catalase hydrogen peroxide. The values of k_4 are 2000 and 470 $\text{M}^{-1} \times \text{sec}^{-1}$ respectively. The data for methanol and ethanol were given previously (1, 6). 0.54 μM of horse blood catalase, 200 μM of hydrogen peroxide, $\lambda = 405 \text{ m}\mu$, 0.01 M phosphate buffer, pH 7.2 (Experiments 372 and 373).

composition of this complex when $a_0 = 0$ (2, 6). For catalase methyl hydrogen peroxide,

$$k_4 a_0 = \frac{[\text{CH}_3\text{OOH}]_0}{[\text{Cat}(\text{OOH})_4] t_{\frac{1}{2}}} \quad (2)$$

where $[\text{CH}_3\text{OOH}]_0$ is the initial concentration, $[\text{Cat}(\text{OOH})_4]$ is the maximum concentration of Complex I, and $t_{\frac{1}{2}}$ is the half time of the "cycle" of Complex I (7).

All experiments were carried out at pH 7.2 and 25°.

Velocity Constants for Reaction of Catalase Hydrogen Peroxide with Nitrite and Formate—The addition of nitrite or formate to catalase hydrogen peroxide causes a decrease of $t_{\frac{1}{2}}$, and the increase in the values of $k_4 a_0$ with increasing a_0 is plotted on a logarithmic scale in Fig. 1. Previous data on methanol and ethanol are included for comparison (2). By avoiding the

limitations of this method studied previously (6), k_4a_0 is found to increase linearly with a_0 as indicated by the 45° straight line of Fig. 1. Thus nitrite and formate react with catalase hydrogen peroxide in a second order reaction as in the case of the alcohols. The reaction velocity constants are 2000 and 470 $\text{M}^{-1} \times \text{sec}^{-1}$ respectively at pH 7.2. These values for formate and nitrite are, however, the apparent reaction velocity constants, since other experiments (unpublished data) show that the acids and not the ions react with the catalase-peroxide complexes. In this

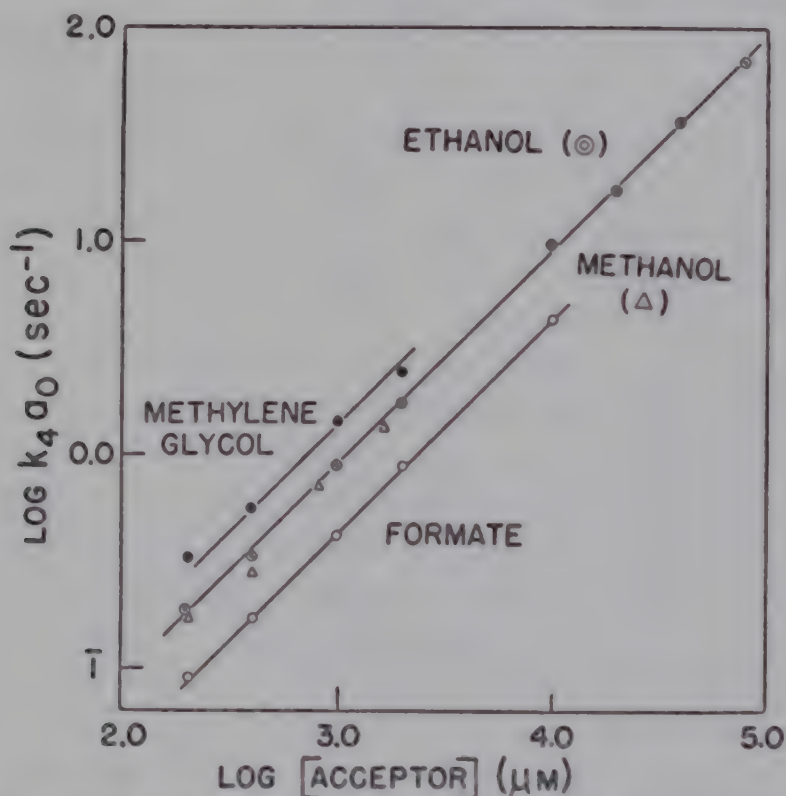


FIG. 2. The relation between reaction velocity and concentration for the reaction of methylene glycol and formate with catalase methyl hydrogen peroxide. The values of k_4 are 1400 and 460 $\text{M}^{-1} \times \text{sec}^{-1}$ respectively. New data on ethanol are given over a wide range of concentrations. The data with methanol were given previously (7). 0.54 μM of horse blood catalase, 10 μM of methyl hydrogen peroxide, 0.01 M phosphate buffer, pH 7.2, $\lambda = 405 \text{ m}\mu$ (Experiment 374).

paper the reaction velocity constants are evaluated on the basis of total formate and nitrite concentrations in order that the data would be comparable with the data on ethanol, etc. Furthermore, these are the reaction velocities that would occur under physiological conditions.

Velocity Constants for Reaction of Catalase Methyl Hydrogen Peroxide with Methylene Glycol² and Formate—Both methylene glycol and formate accelerate the decomposition of the primary catalase-methyl hydrogen peroxide complex. The increase of k_4a_0 with a_0 is computed according to

² In dilute solutions, formaldehyde is nearly completely in the form of methylene glycol, $\text{CH}_2(\text{OH})_2$ (8).

equation (2) and is plotted on a logarithmic scale in Fig. 2, together with previous data on methanol and extended data on ethanol. Here, too, the 45° straight lines verify a second order reaction of enzyme-substrate complex with acceptor. The numerical values of the velocity constants are $1400 \text{ M}^{-1} \times \text{sec.}^{-1}$ for methylene glycol and $460 \text{ M}^{-1} \times \text{sec.}^{-1}$ for formate. As in the case of methanol and ethanol, identical values of k_4 for formate are measured from the kinetics of catalase hydrogen peroxide or catalase methyl hydrogen peroxide, in spite of the rather different experimental conditions and the method of calculation.

Evidence for Ternary Complex in Reaction of Catalase Methyl Hydrogen Peroxide and Ethanol—Previous tests showed that the reaction of catalase hydrogen peroxide and ethanol is unsuitable for accurately measuring the value of k_4 at very large ethanol concentrations ($>4 \text{ mM}$) (6). The phenomenon that limits the accuracy with catalase hydrogen peroxide is not present in the reaction of catalase methyl hydrogen peroxide with ethanol: equation (2) is theoretically valid for any value of a_0 as long as $[\text{Cat}(\text{OOH})_4]$ has a measurable value. In order to fulfil the latter requirement, the methyl hydrogen peroxide concentration is increased in proportion to the ethanol concentration. In the experiments of Fig. 2 the value of $[\text{CH}_3\text{OOH}]/[\text{C}_2\text{H}_5\text{OH}]$ was 0.01 and satisfactory measurements were obtained up to 80 mM of ethanol. The values of $k_4 a_0$ computed from equation (2) increase linearly with the ethanol concentration and indicate that the reaction of catalase methyl hydrogen peroxide with ethanol is of the second order (k_4 is constant) between 0.2 and 80 mM ethanol. There is no measurable decrease of k_4 at the highest ethanol concentration, and, therefore, no evidence of the existence of a ternary catalase-methyl hydrogen peroxide-ethanol complex is afforded by these data.

These data indicate that the mean value of k_4 for the reaction of ethanol or methanol with catalase methyl hydrogen peroxide is $910 \text{ M}^{-1} \times \text{sec.}^{-1}$, and agrees to within the experimental error with the value computed by equation (1) and given in Fig. 1 ($1000 \text{ M}^{-1} \times \text{sec.}^{-1}$).

Reactions of Methylene Glycol with Catalase Hydrogen Peroxide and Nitrite with Catalase Methyl Hydrogen Peroxide—The data above show that methanol, ethanol, and formate react with catalase peroxides at nearly identical velocities regardless of whether hydrogen peroxide or methyl hydrogen peroxide is attached to catalase. It is, therefore, reasonable to conclude that these reactions follow similar mechanisms. Two possible exceptions to this rule have been found.

The addition of methylene glycol to catalase hydrogen peroxide accelerates its rate of decomposition, and below 100 μM of methylene glycol a linear relation between this rate and the methylene glycol concentration is obtained, as shown in Fig. 3. However, the value of k_4 computed from the

slope of this line is $2900 \text{ M}^{-1} \times \text{sec.}^{-1}$ compared with $1400 \text{ M}^{-1} \times \text{sec.}^{-1}$ measured from the kinetics of catalase methyl hydrogen peroxide in the presence of methylene glycol. In the latter reaction, the value of k_4 is con-

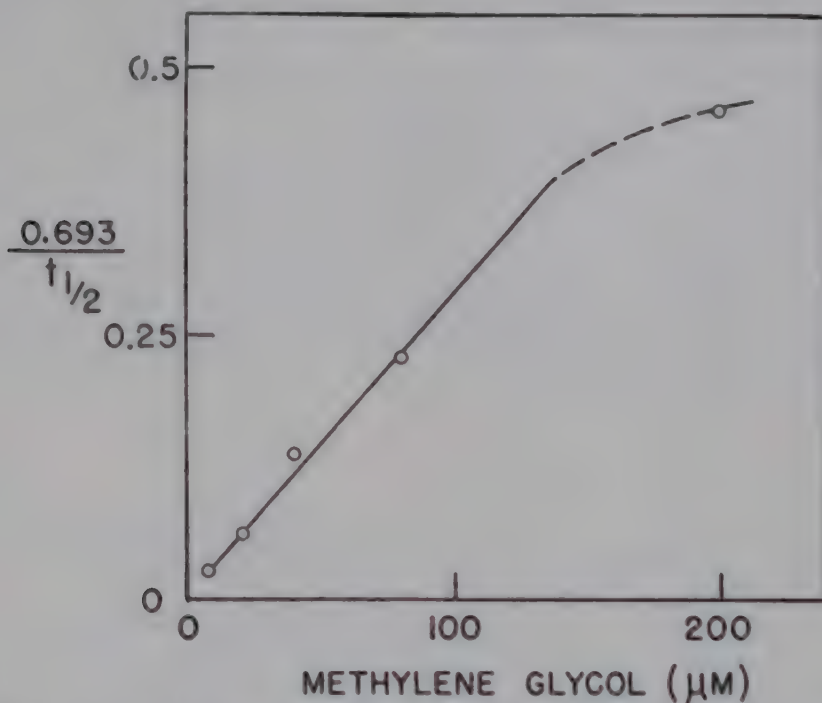


FIG. 3. The relation between reaction velocity and concentration for the reaction of catalase hydrogen peroxide and dilute methylene glycol. The initial value of k_4 is $2900 \text{ M}^{-1} \times \text{sec.}^{-1}$, $0.54 \mu\text{M}$ of horse liver catalase, $4 \mu\text{M}$ of hydrogen peroxide, pH 7.2, 0.01 M phosphate, $\lambda = 405 \text{ m}\mu$ (Experiment 397).

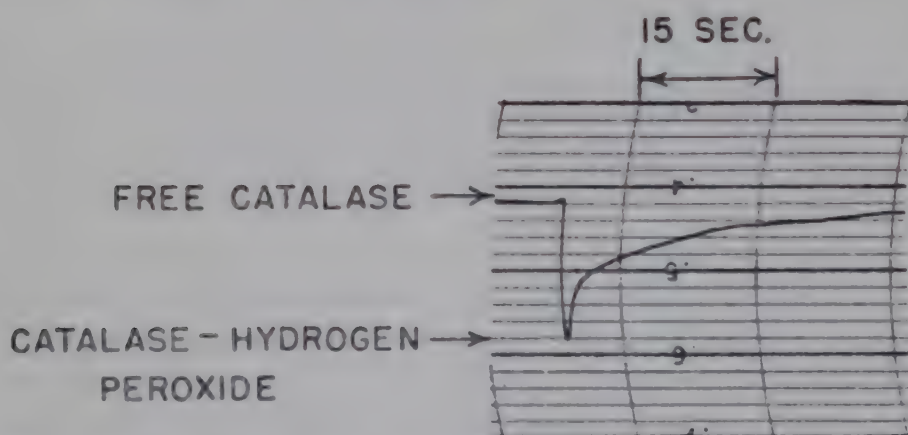


FIG. 4. The reaction kinetics of catalase hydrogen peroxide in the presence of methylene glycol, showing the progressive decrease of reaction velocity. $0.54 \mu\text{M}$ of horse liver catalase, $20 \mu\text{M}$ of hydrogen peroxide, $800 \mu\text{M}$ of methylene glycol, pH 7.2, 0.01 M phosphate, $\lambda = 405 \text{ m}\mu$ (Experiment 397).

stant over a wide range of methylene glycol concentration (tested up to 2 M; see Fig. 2). But in the reaction kinetics of catalase hydrogen peroxide with methylene glycol, a striking change occurs at higher concentrations of the latter, as illustrated in Figs. 3 and 4.

Whereas the kinetics of decomposition of catalase hydrogen peroxide in the presence of other acceptors, *e.g.* ethanol (see Chance (2), Fig. 12), follow a first order or simple exponential curve with a high accuracy, the reaction kinetics illustrated in Fig. 4 are complex; a progressive decrease of the reaction velocity occurs. Also the values of k_4 computed from the initial phases of Fig. 4 are less than those measured in the presence of smaller methylene glycol concentrations.

Since the nature of the kinetics of Fig. 4 suggests that a progressive inhibition of catalase is occurring, catalase was first mixed with methylene glycol and then allowed to react with hydrogen peroxide in the rapid flow apparatus. However, the same type of reaction kinetics was measured. This would indicate that the inhibition is not caused by a direct reaction of catalase and methylene glycol.

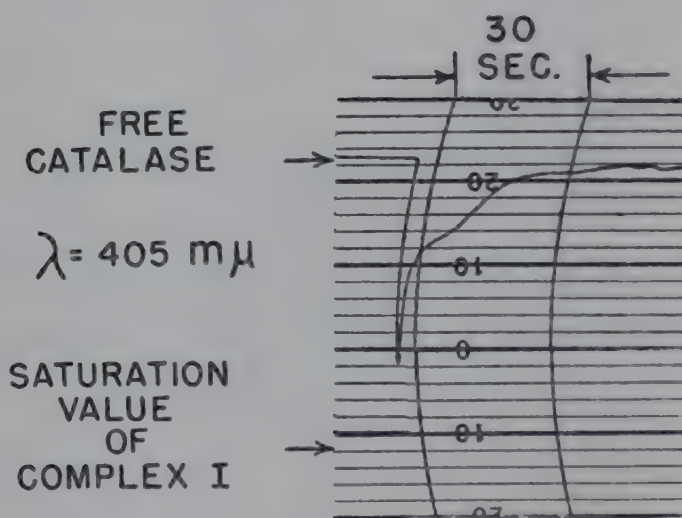


FIG. 5. The reaction kinetics of catalase methyl hydrogen peroxide in the presence of nitrite. $0.54 \mu\text{M}$ of horse erythrocyte catalase, $400 \mu\text{M}$ of nitrite, $10 \mu\text{M}$ of methyl hydrogen peroxide, $\lambda = 405 \text{ m}\mu$, pH 7.2, 0.01 M phosphate (Experiment 374f).

These data suggest that the course of the oxidation of methylene glycol by catalase hydrogen peroxide may differ from that by catalase methyl hydrogen peroxide and, in the former case, an inhibition of the reaction occurs at higher methylene glycol concentrations. In view of these complications, the value of $k_4 = 2900 \text{ M}^{-1} \times \text{sec}^{-1}$ computed from Fig. 3 must be regarded as a preliminary value. It is probable that methylene glycol will not react satisfactorily in coupled oxidations.

A possibly related phenomenon occurs in the reaction of catalase methyl hydrogen peroxide and nitrite, as illustrated in Fig. 5. The decomposition of the catalase-methyl hydrogen peroxide complex, instead of going smoothly to completion, abruptly decelerates, and slowly goes to completion. It is considered premature to compute a value of k_4 from the earlier portions of the reaction kinetics.

Reaction of Catalase Peroxide with Ascorbic Acid—An increase in the velocity of disappearance of catalase hydrogen peroxide was previously found to occur upon the addition of dilute ascorbic acid (2). Since ascorbic acid was later found to accelerate the formation of the inactive secondary catalase-hydrogen peroxide complex (9), the kinetics of catalase in the presence of ascorbic acid have been reinvestigated. The records of Fig. 6 confirm the previous result that the addition of dilute ascorbic acid accelerates the disappearance of catalase hydrogen peroxide; the value of t_1 in record *B* is less than that in record *A* of Fig. 6 and the value of k_4 computed according to equation (1) is $330 \text{ M}^{-1} \times \text{sec.}^{-1}$ in accordance with the previous value. However, there is a significant difference between the magnitude of the optical density changes shown in records *A* and *B*. In records *A* and *B*,

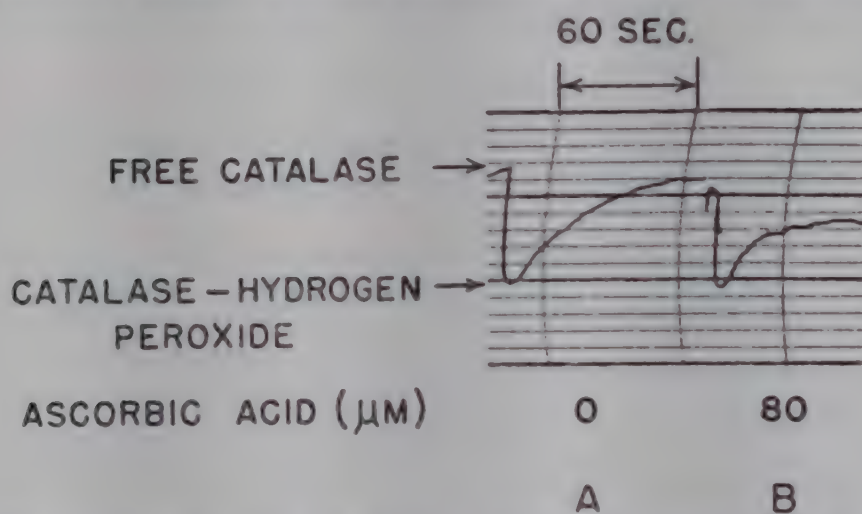


FIG. 6. The reaction kinetics of catalase hydrogen peroxide in the absence (*A*) and presence (*B*) of ascorbic acid, showing the acceleration of the decomposition of the complex and the reduction of the optical density change in (*B*). $0.54 \mu\text{M}$ of horse liver catalase, $2 \mu\text{M}$ of hydrogen peroxide, $8 \mu\text{M}$ of ethanol, ascorbic acid as shown, 0.01 M phosphate, $\text{pH } 7.2$, $\lambda = 405 \text{ m}\mu$ (Experiment 400).

the same optical density (that of catalase hydrogen peroxide) is measured immediately after the reactants are injected into the capillary observation tube of the rapid flow apparatus. Thereafter, the tracing of record *A* rises exponentially 6 scale divisions to the optical density of free catalase, while that of record *B* rises only 3.5 scale divisions, and not to the optical density of free catalase. Thus a portion of the primary complex is converted into the secondary complex in the presence of ascorbic acid and not into free catalase. This complicating feature obscures the measurements of the kinetics of the reaction of ascorbic acid with the primary catalase-hydrogen peroxide complex.

The effect of ascorbic acid upon the rate of disappearance of catalase methyl hydrogen peroxide has therefore been studied and scarcely any increase is measured. Apparently catalase methyl hydrogen peroxide does not react with ascorbic acid at a measurable rate.

It is therefore concluded that the acceleration of the disappearance of catalase hydrogen peroxide in the presence of dilute ascorbic acid is associated with the formation of the secondary complex and does not represent a peroxidatic reaction similar to that found with alcohols, etc.

DISCUSSION

On the basis of the high reactivity of catalase peroxides towards methanol, methylene glycol, formate, and ethanol compared with their inactivity towards acetaldehyde and acetic acid (see Table I), the general requirements for a catalase acceptor are a hydrogen atom and a hydroxyl group attached to a carbon atom (7). Since catalase peroxides also rapidly oxidize hydrogen peroxide, the carbon atom can apparently be replaced by an oxygen atom. And the oxidation of nitrite by catalase hydrogen per-

TABLE I

Reactivities of Various Substances towards Catalase Peroxides

A value of k_4 greater than $10 \text{ M}^{-1} \times \text{sec.}^{-1}$ is significant under the experimental conditions. pH 7.0, $\sim 1 \mu\text{M}$ of catalase.

Experiment No.	Substance	Concentration	Peroxide	Concentration	$k_4 (\text{M}^{-1} \times \text{sec.}^{-1})$
		<i>mM</i>		<i>μM</i>	
81	Acetone	14	H_2O_2	200	12
81	"	90	"	200	8
109	Acetate	10	"	200	<2
109	Acetaldehyde	4	"	200	<2
113	Acetoacetate	2.5	"	400	2
162	Pyruvate	14	CH_3OOH	4.4	1
287	Ethyl mercaptan	1.0	H_2O_2	200	90

oxide shows that nitrogen is suitable as a central atom. However, nitrite

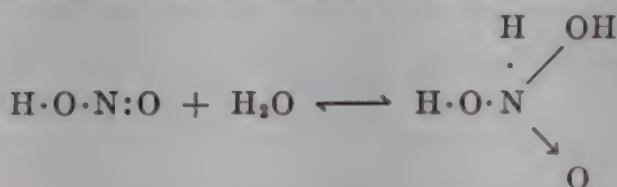
has two tautomeric forms, $\text{H} \cdot \text{O} \cdot \text{N} : \text{O}$ and $\text{H} - \text{N} \begin{array}{c} \nearrow \text{O} \\ \searrow \text{O} \end{array}$ (for a discussion, see Sidgwick (10)). Therefore, the reactivity of catalase hydrogen peroxide

towards ethyl nitrite, $\text{C}_2\text{H}_5\text{O} \cdot \text{N} : \text{O}$, and nitroethane, $\text{C}_2\text{H}_5 - \text{N} \begin{array}{c} \nearrow \text{O} \\ \searrow \text{O} \end{array}$, has

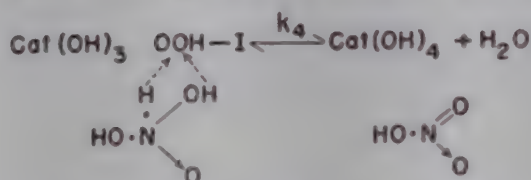
been tested. The latter is apparently inactive ($k_4 \sim 17 \text{ M}^{-1} \times \text{sec.}^{-1}$) and the former is very active (k_4 , preliminary value, $\sim 2800 \text{ M}^{-1} \times \text{sec.}^{-1}$). Thus the form $\text{H} \cdot \text{O} \cdot \text{N} : \text{O}$ is probably the reactive one. In the experiments with ethyl nitrite, it is probable that considerable hydrolysis into ethanol

and nitrous acid occurred. On the other hand, there is no reason to doubt that direct oxidation of ethyl nitrite could occur ((10) p. 5).

In the discussion of nitrous acid, Sidgwick (10) presents a modification of von Baeyer and Villiger's (11) hypothesis for the reactions of nitrous acid. In water solutions there is a hydration of nitrous acid.



In this case it is possible to write the same mechanism for the oxidation of nitrous acid as was given for the oxidation of alcohols, etc., by catalase hydrogen peroxide; thus the hydrated form would be expected to react:



Thus the definition of catalase specificity can be extended to include a hydrogen atom and a hydroxyl group attached to a nitrogen atom.

The resemblance between the structures of nitrous and phosphorous acids has led us to try the latter, but without successful results to date.

On the other hand, the decomposition of catalase hydrogen peroxide in the presence of ascorbic acid is not a second order reaction and does not represent a peroxidatic oxidation of ascorbic acid as previously stated (2). And on the basis of catalase specificity, an oxidation of ascorbic acid by catalase hydrogen peroxide would not be expected because the oxidizable enediol group of ascorbic acid ($-\text{C}=\text{C}-$) has no hydrogen atom at-



tached to the carbon atom.

The numerical values of k_4 for the reaction with nitrite and methylene glycol are the largest values measured for a catalase acceptor, 2000 and $1400 \text{ M}^{-1} \times \text{sec.}^{-1}$, respectively, and confirm the general rule that catalase peroxides react more rapidly with smaller molecules. The reactions of longer chain alcohols and glycols with catalase peroxides proceed very slowly (n -propyl = 17 (2) and ethylene glycol $\sim 19 \text{ M}^{-1} \times \text{sec.}^{-1}$).

The nearly identical reactivity of catalase hydrogen peroxide and catalase methyl hydrogen peroxide towards ethanol and formate is indicated by the respective values of $k_4 = 1000$ and 910 and 470 and 460 $\text{M}^{-1} \times \text{sec.}^{-1}$ at 25° . The discrepancies are within the experimental error. Keilin and Hartree (personal communication) have found that formate promotes a typical coupled oxidation, in which carbon dioxide is produced.

In the reactions of nitrite and methylene glycol with catalase peroxides apparent differences in reactivity have been found, depending upon whether hydrogen peroxide or methyl hydrogen peroxide is used as a substrate. In the first case, however, direct experimental data show that a progressive inhibition of catalase occurs when hydrogen peroxide is used as a substrate instead of methyl hydrogen peroxide. A similar effect is recorded when catalase methyl hydrogen peroxide reacts with nitrite. It is possible that the course of the reaction may differ depending upon whether hydrogen peroxide or methyl hydrogen peroxide is used.

SUMMARY

1. The velocity constant for the reactions of catalase hydrogen peroxide with nitrite is $2000 \text{ M}^{-1} \times \text{sec.}^{-1}$ and with formate is $470 \text{ M}^{-1} \times \text{sec.}^{-1}$ at pH 7.2.

2. The velocity constant for the reaction of catalase methyl hydrogen peroxide with methylene glycol is $1400 \text{ M}^{-1} \times \text{sec.}^{-1}$ and with formate $460 \text{ M}^{-1} \times \text{sec.}^{-1}$ at pH 7.2.

3. The reaction of catalase methyl hydrogen peroxide with ethanol is of the second order between 0.2 and 80 mm of ethanol; no decrease of the velocity constant occurs at a turnover number of 80 sec.^{-1} (in 80 mm of ethanol).

4. The reactions of catalase hydrogen peroxide with methylene glycol and catalase methyl hydrogen peroxide with nitrite show a progressive inhibition of the activity of the catalase peroxides and accurate values of the velocity constants for these reactions have not been obtained.

5. The acceleration of the disappearance of the primary catalase hydrogen peroxide in the presence of dilute ascorbic acid is associated with the formation of the secondary complex and does not represent a peroxidatic reaction similar to that found with alcohols, etc.

BIBLIOGRAPHY

1. Keilin, D., and Hartree, E. F., *Biochem. J.*, **39**, 293 (1945).
2. Chance, B., *Acta. chem. Scand.*, **1**, 236 (1949).
3. Chance, B., *J. Biol. Chem.*, **180**, 947 (1949).
4. Heppel, L. A., and Porterfield, V. T., *J. Biol. Chem.*, **178**, 549 (1949).
5. Chance, B., *J. Biol. Chem.*, **179**, 1331 (1949).
6. Chance, B., *J. Biol. Chem.*, **182**, 643 (1950).
7. Chance, B., *J. Biol. Chem.*, **179**, 1341 (1949).
8. Walker, J. F., Formaldehyde, American Chemical Society Monograph series, New York (1944).
9. Chance, B., *Biochem. J.*, in press.
10. Sidgwick, N. V., The organic chemistry of nitrogen, edited by Taylor, T. W. J., and Baker, W., Oxford (1937).
11. von Baeyer, A., and Villiger, V., *Ber. chem. Ges.*, **34**, 755 (1934).

EFFECT OF DIETARY TRYPTOPHAN IMBALANCE UPON THE METABOLISM OF TRYPTOPHAN AND NICOTINIC ACID IN MAN*

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Growth of animals receiving diets low in protein and nicotinic acid may be retarded by the addition of corn products (1-4), of certain amino acids (5-9), or of proteins deficient in tryptophan (8-11). This inhibition, which is obtained with diets relatively deficient in tryptophan, can be overcome by the administration of nicotinic acid or of tryptophan (1-11). It has been suggested that this dietary amino acid imbalance is an important pellagragenic factor (5-11).

In the present experiments a dietary amino acid imbalance (tryptophan-deficient) was created in man by the addition of gelatin or glycine to an otherwise constant diet, and the effect upon the excretion of nicotinic acid and tryptophan metabolites was studied. Since these experiments have been carried out, other workers have shown that the addition of gelatin to the casein diet of rats had little effect upon N^1 -methylnicotinamide (N^1 -Me) excretion, although the dietary change inhibited the growth of the animals (12, 13).

Previous work on man has shown that the replacement of part of the wheat in a diet by corn products resulted in a decreased excretion of N^1 -Me (14, 15). It could not be determined from the data whether this was due in part to the presence in corn of an inhibitory agent (16) which interfered with the metabolism of tryptophan or nicotinic acid, or whether it was due entirely to the lower tryptophan and protein content of the corn diet compared with the wheat diet. In the present studies on the effect of corn products, the tryptophan and protein levels of the corn and wheat diets were maintained at more comparable levels.

EXPERIMENTAL

The subjects were ward patients similar to those employed in previous studies (14, 15) and were maintained in a separate metabolism ward. The

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corn and wheat diets used with all of the subjects, except Subjects 21 and 22, have been described previously (15), and were planned to provide about 2470 and 2400 calories, 38 and 43 gm. of protein, 380 and 500 mg. of tryptophan,¹ and 5.5 and 6 mg. of nicotinic acid per day, respectively. The diets differed only in regard to their content of corn and wheat products.² The wheat diet supplied 248 gm. of unenriched wheat products per day, whereas in the corn diet 180 gm. of this were replaced by 190 gm. of corn-meal and grits. In the *special* corn diet employed for Subjects 21 and 22, a smaller part of the wheat was replaced by about twice its weight of corn products; thus this diet contained almost as much tryptophan as did the wheat diet. The diets were varied slightly to suit each of the subjects, and the recorded average food intakes are given in Tables I to V. Supplements to the diets were divided between dinner and supper and were not included in the calculated food intake.

The wheat and corn products and the casein used in these studies were analyzed for tryptophan by a microbiological method (15), after alkaline hydrolysis in the presence of cysteine (17). The tryptophan content of these foods in terms of mg. per gm. was as follows: white flour 1.17, cream of wheat 1.23, corn-meal 0.45, corn grits 0.43, and casein (Labco, vitamin-free) 10.5.

24 hour urines were collected in amber bottles containing 15 ml. of glacial acetic acid, stored in the refrigerator, and pooled in 48 hour periods for measurement of creatinine, nitrogen, *N*¹-Me, and 4-pyridoxic acid by chemical methods and tryptophan and nicotinic acid by microbiological assay. The analyses were conducted by methods previously described (15), except that *N*¹-Me was measured by the acetone fluorometric method (18) and nitrogen by the Kjeldahl procedure. Creatinine values are not reported, but were determined in all urines to check the completeness of collection.

The excretion of two tryptophan metabolites, which have been recently described (15, 19, 20), was also measured in several of the experiments. Tryptophan analyses were performed on urine samples before and after ether extraction at pH 4 in order to measure an ether-soluble tryptophan-like compound which can replace L-tryptophan for growth of *Lactobacillus arabinosus* (15). The nicotinic acid content of the urine was assayed before and after autoclaving for 1 hour in 1 N sulfuric acid (15). The in-

¹ The tryptophan content of these diets has been recalculated, with values obtained by analysis of the corn and wheat products.

² The unfortified corn products used in these diets were generously provided by the Quaker Oats Company, through the courtesy of Dr. F. N. Peters, and the unenriched wheat products by General Mills, Inc., through the courtesy of Dr. F. C. Hildebrand and Dr. H. S. Faulkner.

crease in the nicotinic acid value after acid autoclaving is probably due to the decarboxylation of quinolinic acid (20). This substance has been isolated from the urine of rats which had received DL-tryptophan (21). Under the conditions described, about one-fifth of the quinolinic acid is changed to nicotinic acid, whereas half maximal conversion is obtained by autoclaving about 4 hours (15).

Results

Two subjects (Subject 9, B. G., female, age 36, weight 58 kilos; Subject 10, H. U., male, age 28, weight 67 kilos) were maintained on the wheat diet for 40 days. During the second 10 day period, 20 gm. of gelatin³ were added to the daily diet and in the third 10 day period, 20 gm. of glycine (Merck). Table I shows the excretion of nitrogen, nicotinic acid, "quinolinic acid," *N*¹-Me, tryptophan, and 4-pyridoxic acid by these subjects during the experimental periods. The increase in nicotinic acid value found after acid autoclaving over that obtained before autoclaving represents about one-fifth of the "quinolinic acid" in the urine.

There were no apparent changes in excretion of nicotinic acid, "quinolinic acid," or *N*¹-Me by either subject when gelatin or glycine was added to the diet (Table I). The removal of the glycine from the diet was accompanied by a slight decrease in excretion of these compounds. The findings are similar to those on rats, in which the addition of 12 per cent gelatin to a 12 per cent casein diet (12) or of 6 per cent gelatin to a 10 per cent casein diet (13) resulted in the same or higher *N*¹-Me excretion than in the unsupplemented diets, although the added gelatin inhibited growth of the animals and lowered the extra *N*¹-Me excretion after a test dose of tryptophan.

When 20 gm. of gelatin, containing 3 gm. of nitrogen, were added to the diets, the subjects excreted in the urine an average of 1.4 and 1.6 gm. more nitrogen than they did during the basal wheat period (Table I). The tryptophan excretion was either about the same or slightly lower than during the basal period. The replacement of the gelatin by glycine, which contributed 3.7 gm. of nitrogen to the diet, resulted in an average excretion of 3.3 and 3.5 gm. of nitrogen over the original basal value. This was accompanied by an increased excretion of urinary tryptophan by both subjects (Table I). The addition of the gelatin or the glycine had no significant effect upon the excretion of 4-pyridoxic acid, although pyridoxine is known to be necessary for protein metabolism (22). After the glycine was removed from the diets, there was a slight increase in excretion of 4-pyridoxic acid by both subjects.

³ The gelatin used in these studies was generously supplied by the Knox Gelatine Protein Products, Inc., through the courtesy of Dr. D. Tourtellotte.

When the corn diet was used in a similar experiment (Subject 12, Z. M., age 38, weight 64 kilos), the creation of an amino acid imbalance by the addition of gelatin or glycine led to little change in excretion of nicotinic acid compounds (Table II). In the last part of the experiment, the inclusion of 200 mg. of L-tryptophan⁴ with 20 gm. of gelatin led to a slight in-

TABLE I

Effect of Administration of Gelatin and Glycine upon Daily Excretion of Nicotinic Acid and Related Compounds

Diet	Day No.	Nitrogen		Nicotinic acid				N ¹ -Me		Trypto- phan		4-Pyridoxic acid	
				Before acid autoclaving		After acid autoclaving							
		Sub- ject 9	Sub- ject 10	Sub- ject 9	Sub- ject 10	Sub- ject 9	Sub- ject 10	Sub- ject 9	Sub- ject 10	Sub- ject 9	Sub- ject 10	Sub- ject 9	Sub- ject 10
		gm.	gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Wheat*	1- 2	5.3	7.6	0.9	1.0	1.7	2.0	2.1	3.3	15	9	0.8	1.4
	3- 4	5.0	6.7	0.7	0.9	1.5	1.8	2.0	3.0	17	9	0.9	1.8
	5- 6	4.5	6.3	0.5	0.8	1.1	1.8	1.5	3.2	16	8	1.1	1.7
	7- 8	4.7	7.3	0.5	0.6	1.2	1.3	1.3	3.3	16	9	1.0	2.2
	9-10	4.5	7.0	0.8	0.9	1.6	1.8	1.5	3.5	16	10	1.0	2.3
Wheat + 20 gm. gelatin per day	11-12	5.9	7.6	0.6	0.9	1.2	2.0	1.4	3.3	11	7	1.0	1.3
	13-14	6.0	9.2	0.5	0.7	1.0	1.5	1.4	3.5	13	8	1.2	2.0
	15-16	6.4	7.4	0.7	0.8	1.6	1.6	1.4	2.6	10	6	0.4	1.9
	17-18	6.0	8.9	0.7	0.7	1.4	1.6	1.3	2.9	11	8	1.3	2.1
	19-20	6.8	9.9	0.6	0.6	1.3	1.8	1.5	3.2	12	9	1.3	2.1
Wheat + 20 gm. glycine per day	21-22	8.2	11.3	0.5	0.6	1.5	2.1	1.5	3.5	15	11	0.7	0.9
	23-24	8.3	11.2	0.6	0.8	1.2	1.6	1.3	3.3	18	11	0.9	1.8
	25-26	8.3	9.7	0.6	0.8	1.3	1.7	1.6	3.0	18	10	0.9	1.4
	27-28	8.0		0.5		1.4		1.6		19		1.1	
	29-30	7.9	9.9	0.9	1.0	1.6	1.9	1.6	2.8	20	10	1.3	1.7
Wheat	31-32	5.5	7.1	0.5	1.1	1.1	1.9	1.5	3.1	17	10	2.1	1.8
	33-34	4.7	6.2	0.5	0.8	1.2	1.6	1.5	2.9	15	9	1.9	1.8
	35-36	4.9	5.6	0.5	0.5	1.2	1.3	1.0	2.8	17	10	2.0	2.0
	37-38	4.7	6.0	0.6	0.5	1.2	1.3	1.2	2.8	18	11	2.0	2.0
	39-40	5.1	5.9	0.7	0.5	1.5	1.2	1.0	2.7	18	12	0.9	2.1

* Average intake, Subject 9, 2730 calories, 43 gm. of protein, 5.9 mg. of nicotinic acid; Subject 10, 2520 calories, 41 gm. of protein, 6.1 mg. of nicotinic acid.

crease in the "quinolinic acid" excretion but had no effect upon the level of nicotinic acid or N¹-Me.

The level of tryptophan excretion was decreased when gelatin or glycine was added to the diet, but did not return to the basal level when these supplements were removed (Table II).

⁴ Dr. C. Robertson of the Van Camp Laboratories generously furnished the L-tryptophan used in these experiments.

The average extra nitrogen excretion (1.4 gm. per day) which was found when the gelatin was added to the corn diet was similar to that shown for Subjects 9 and 10 on the wheat diet (Table I). However, the extra nitrogen excreted when glycine was added to the corn diet was somewhat less (about 2.5 to 3.0 gm. average, Table II) than the 3.3 and 3.5 gm. increases

TABLE II

Effect of Administration of Gelatin, Glycine, and Tryptophan upon Daily Excretion of Nicotinic Acid and Related Compounds by Subject 12

Diet	Day No.	Nitrogen	Nicotinic acid		Nl-Me	Tryptophan	4-Pyridoxic acid
			Before acid auto-claving	After acid auto-claving			
		gm.	mg.	mg.	mg.	mg.	mg.
Corn (2670 calories, 41 gm. protein, 6.6 mg. nicotinic acid)	1- 2	5.5	0.5	1.3	1.9	11	0.6
	3- 4	4.9	0.4	1.1	1.6	10	0.9
	5- 6	4.7	0.4	1.3	1.3	11	0.8
	7- 8	4.7	0.4	1.4	1.3	8	0.7
Corn + 20 gm. gelatin per day	9-10	6.0	0.4	1.1	1.2	9	1.0
	11-12						
	13-14	5.9	0.4	1.2	1.3	9	0.8
	15-16	6.6	0.5	1.4	1.4	8	0.8
	17-18	7.2	0.5	1.3	1.3	7	0.7
Corn + 20 gm. glycine per day	19-20	7.0	0.4	1.3	1.3	7	0.7
	21-22	7.7	0.4	1.3	1.4	8	0.9
	23-24	7.4	0.4	1.1	1.4	8	1.0
	25-26	7.2	0.4	1.1	1.5	7	0.7
	27-28	7.7	0.4	1.2	1.5	7	0.9
Corn	29-30	4.3	0.3	1.0	1.3	6	0.9
	31-32	4.6	0.4	1.1	1.3	7	1.0
	33-34	4.8	0.3	1.0	1.4	7	0.9
	35-36	4.5	0.3	1.0	1.1	7	1.0
Corn + 20 gm. gelatin + 200 mg. L-tryptophan per day	37-38	4.6	0.3	1.3	1.2	13	0.8
	39-40	6.2	0.5	1.6	1.2	13	0.5
	41-42	6.3	0.4	1.4	1.2	11	0.5
	43-44	6.6	0.4	1.4	1.2	11	0.7
	45-46	7.0	0.4	1.4	1.2	11	0.6

found with the subjects on the wheat diet. Excretion of 4-pyridoxic acid was not affected by the addition of the gelatin or glycine, but was decreased slightly during supplementation with gelatin and tryptophan.

Since the addition of gelatin or glycine to basal wheat or corn diets had no effect upon the excretion of nicotinic acid compounds by the first three subjects, the addition of 30 gm. of gelatin to the diet of a subject (No. 20, R. R., female, age 30, weight 44 kilos), who was already receiving an added

500 mg. of L-tryptophan per day, was tried. The data in Table III show that the addition of the L-tryptophan to the wheat diet resulted in an increased excretion of N^1 -Me, nicotinic acid, "quinolinic acid," and of tryptophan and that the further supplementation with 30 gm. of gelatin per day for 12 days maintained these same levels of excretion. The nitrogen excretion was increased by about 3 gm. after the addition of this gelatin, which contained 4.5 gm. of nitrogen. The excretion of 4-pyridoxic acid was not changed by the addition of the tryptophan, but appeared to de-

TABLE III

Effect of Administration of L-Tryptophan and Gelatin upon Daily Excretion of Nicotinic Acid and Related Compounds by Subject 20

Diet	Day No.	Nitrogen	Nicotinic acid		N^1 -Me	Tryptophan		4-Pyridoxic acid
			Before acid auto-claving	After acid auto-claving		Before ether extraction	After ether extraction	
		gm.	mg.	mg.	mg.	mg.	mg.	mg.
Wheat (2030 calories, 34 gm. protein, 5.1 mg. nicotinic acid)	1- 2	4.2	0.5	1.0	1.7	8	7	1.5
	3- 4	3.5	0.4	0.9	1.4	8	7	1.6
	5- 6	3.5	0.5	1.0	1.3	6	5	1.3
	7- 8	3.1	0.4	1.0	1.2	6	6	1.2
Wheat + 500 mg. L-tryptophan per day	9-10	3.3	0.4	1.3	1.2	9	5	1.4
	11-12	3.5	0.5	1.3	1.6	9	8	1.4
	14	3.9	0.4	1.3	2.2	11	9	1.3
	15-16	3.7	0.4	1.2	1.8	10	7	1.0
	17-18	4.3	0.6	1.4	2.6	11	11	1.6
	19-20	4.5	0.7	1.6	2.8	11	10	0.9
Wheat + 500 mg. L-tryptophan + 30 gm. gelatin per day	21-22	7.1	0.7	1.5	3.2	11	10	1.6
	23-24	7.1	0.7	1.4	2.4	10	9	0.8
	25-26	7.1	0.7	1.5	2.4	10	9	1.1
	27-28	6.7	0.5	1.1	2.1	12	10	0.8
	29-30	7.3	0.5	1.3	2.8	11	9	0.8
	31-32	6.8	0.6	1.4	2.6	10	9	0.9

crease slightly after the gelatin and tryptophan were added, similar to the results for Subject 12 (Table II).

It appears from the data in these four experiments (Tables I to III) that, in man, the addition to the diet of an incomplete protein or an amino acid which markedly reduces the per cent of tryptophan in the total amino acids available has no effect in short term experiments upon the level of excretion of nicotinic acid compounds in the urine.

Since the addition of gelatin had virtually no effect upon the excretion of nicotinic acid metabolites, 30 gm. of vitamin-free casein (Labco) were

added to the diet of Subject 28 (S. D., female, age 56, weight 60 kilos) to see what effect a large amount of a protein containing tryptophan would have upon the excretion of nicotinic acid and tryptophan compounds (Table IV). Subject 28 later received 315 mg. of L-tryptophan (equivalent to that found by analysis in the casein) and 30 gm. of gelatin as a supplement to the diet. Table IV shows that the addition of the casein had no effect upon the excretion of nicotinic acid, "quinolinic acid," or N^1 -Me, but led to a small increase in tryptophan excretion and an average increase of 2.2 gm. of nitrogen (of the 4.5 gm. present in the casein). The excretion of N^1 -Me and of tryptophan was slightly decreased when the casein supplement was removed. The addition of tryptophan and gelatin to the diet (Days 33 to 42) restored the N^1 -Me and tryptophan excretion to approximately that found on the casein-supplemented diet and led to small increases in excretion of nicotinic acid and "quinolinic acid." The average nitrogen excretion was 2.8 gm. higher (of the 4.5 gm. of nitrogen in the gelatin) than in the previous basal period (Table IV).

When the gelatin was removed from the diet, leaving the extra tryptophan (Days 43 to 51), there was a small increase in excretion of N^1 -Me, virtually no change in excretion of nicotinic acid, "quinolinic acid," or tryptophan, and a drop in nitrogen excretion to a level slightly below that of the previous basal period (Table IV). After the tryptophan supplement was removed (Days 52 to 59) there was a slight decrease in the excretion of tryptophan, N^1 -Me, nicotinic acid, and "quinolinic acid" (Table IV). None of the supplements throughout the experiment had any significant effect upon the difference between the tryptophan values found before and after ether extraction of the urine.

In previous experiments it has been shown that the substitution of part of the wheat products in the diet by an equal weight of corn products led to a decreased excretion of N^1 -Me (15). The lowering of the tryptophan content of the diet by this substitution may have been responsible for these findings. The results may also have been due, in part, to the presence of an inhibitory substance in corn (16). Since the experiments described above in the present report (Tables I to IV) show that the addition of extra protein (and calories) had no significant effect on N^1 -Me excretion, the relationship between corn and wheat diets was reinvestigated with extra corn in the diets so that the tryptophan content of the wheat and corn diets would be comparable.

For 10 days two female subjects (Subject 21, M. J., age 46, weight 47 kilos; Subject 22, L. B., age 43, weight 64 kilos) received the wheat diet, which was then replaced by the special corn diet described above. However, the subjects did not consume all of the corn diet which was offered to them, and the amount which they did eat supplied about 40 to 50 mg.

less tryptophan per day than did the wheat diets. Since similar changes in urinary excretion were found with both subjects, the results on only one of the subjects (No. 21) are presented in Table V.

TABLE IV
Effect of Administration of Casein, Gelatin, and L-Tryptophan upon Daily Excretion of Nicotinic Acid and Related Compounds by Subject 28

Diet	Day No.	Nitrogen	Nicotinic acid		N ¹ -Me	Tryptophan	
			Before acid auto-claving	After acid auto-claving		Before ether extraction	After ether extraction
		gm.	mg.	mg.	mg.	mg.	mg.
Wheat (2000 calories, 32 gm. protein, 5.1 mg. nicotinic acid)	1- 2		0.6	1.1	2.6	14	14
	3- 4	4.9	0.6	1.3	2.5	14	13
	5- 6	4.9	0.5	1.1	2.0	13	12
	7- 8	4.6	0.5	0.8	2.6	12	11
	9-10	4.3	0.5	1.0	2.5	12	12
Wheat + 30 gm. casein per day	11-12	6.5	0.4	1.0	2.7	16	15
	13-14	6.3	0.5	1.0	2.2	13	13
	15-16	6.5	0.5	1.0	2.3	14	13
	17-18	7.1	0.5	1.0	2.2	14	13
	19-20	7.9	0.5	1.0	2.4	16	15
Wheat	21-22	4.7	0.4	0.8	1.8	11	10
	23-24	4.9	0.4	0.9	2.2	13	10
	25-26	4.9	0.5	0.8	2.0	12	10
	27-28	5.0	0.5	1.1	1.7	14	12
	29-30	4.7	0.4	1.1	1.9	13	11
	31-32	4.4	0.5	1.1	1.5	12	12
	33-34	6.1	0.5	1.3	1.5	15	14
Wheat + 30 gm. gelatin + 315 mg. L-tryptophan per day	35-36	7.4	0.6	1.4	2.0	17	16
	37-38	7.9	0.7	1.4	2.3	15	14
	39-40	8.0	0.6	1.5	2.1	16	15
	41-42	8.4	0.5	1.3	2.2	17	16
	43-44	5.6	0.5	1.5	2.0	15	15
Wheat + 315 mg. L-tryptophan per day	45-46	4.4	0.5	1.4	2.2	14	14
	47-48	4.4	0.4	1.2	2.8	16	16
	49-50	4.1	0.5	1.4	2.8	15	15
	51	4.1	0.5	1.4	3.0	13	13
	52-53	4.1	0.3	0.8	2.4	9	8
Wheat	54-55	4.4	0.5	1.3	2.1	14	13
	56-57	3.9	0.5	1.2	1.7	11	10
	58-59	3.6	0.4	1.1	1.4	10	10

The excretion of N¹-Me by both subjects was decreased by the change from the wheat to the corn diet and was subsequently increased by the return to the wheat diet (Table V). In Subject 22 the “quinolinic acid” excretion showed similar changes. The tryptophan excretion by both

subjects was decreased slightly by the change to the corn diet and stayed at this lower level when the wheat diet was resumed (Table V). Since there was a difference of only 40 to 50 mg. in the tryptophan content of the wheat and corn diets, the changes in N^1 -Me excretion suggest that factors in corn other than its low tryptophan content may have an effect upon nicotinic acid metabolism.

TABLE V

Effect of Substitution of Special Corn Diet for Wheat Diet upon Daily Excretion of Nicotinic Acid and Related Compounds by Subject 21

Diet*	Day No.	Nitrogen	Nicotinic acid		N^1 -Me	Tryptophan	4-Pyridoxic acid
			Before acid auto-claving	After acid auto-claving			
		gm.	mg.	mg.	mg.	mg.	mg.
Wheat (2040 calories, 32 gm. protein, 5.0 mg. nicotinic acid)	1- 2	4.4	1.1	1.9	2.3	8	0.3
	3- 4	4.2	1.0	1.7	2.1	8	0.4
	5- 6	4.9	1.0	1.8	2.0	7	0.3
	7- 8	4.4	0.9	1.8	2.0	7	0.6
	9-10	4.8	0.9	1.7	2.0	7	0.3
Corn, special (2450 calories, 33 gm. protein, 5.1 mg. nicotinic acid)	11-12	4.2	0.9	1.7	1.6	6	0.8
	13-14	4.0	0.7	1.7	1.4	6	0.8
	15-16	4.3	0.7	1.7	1.5	6	0.9
	17-18	4.0	0.8	1.5	1.4	5	0.9
	19-20	3.8	0.7	1.6	1.3	6	0.8
Wheat	21-22	3.5	0.7	1.5	1.5	6	0.8
	23-24	3.9	0.8	1.5	1.7	6	0.3
	25-26	3.7	0.8	1.4	1.8	6	0.3
	27-28	4.0	0.7	1.5	1.9	7	0.4
	29-30	4.0	0.6	1.6	1.8	6	0.6

* The wheat diet contained approximately 430 mg. of tryptophan, and the corn diet approximately 380 mg. of tryptophan.

The excretion of 4-pyridoxic acid was significantly higher while the subjects received the corn diet than when they consumed the wheat diet (Table V). This increase may be related to the difference in tryptophan, protein, or pyridoxine content of the diets, or to the presence of substances in the food which interfere with the accurate measurement of 4-pyridoxic acid in urine.⁵

DISCUSSION

The addition of a large amount of glycine or of a tryptophan-deficient protein (gelatin) to diets low in protein, tryptophan, and nicotinic acid had no discernible effect in short term experiments upon the level of uri-

⁵ Sarett, H. P., and DiLeo, E., unpublished data.

nary excretion of nicotinic acid compounds by man (Tables I to III). It is possible that the tryptophan imbalance was not severe enough or of long enough duration to affect the N^1 -Me excretion. Since the N^1 -Me excretion by some of the subjects was rather low during the basal period, a decrease might not have been readily apparent. However, when the N^1 -Me excretion was increased slightly by the incorporation of 500 mg. of L-tryptophan per day into the diet (Table III), the addition of 30 gm. of gelatin to the 34 gm. of protein in the diet also failed to alter the N^1 -Me excretion in 12 days.

Although this dietary amino acid imbalance did not affect the urinary excretion of the compounds measured, it may have had some effect upon tryptophan and nicotinic acid metabolism in man. In rats the addition of 12 per cent gelatin to a 12 per cent casein diet or of 6 per cent gelatin to a 10 per cent casein diet had no effect upon the excretion of N^1 -Me, although it inhibited growth of the animals during a 4 week period and lowered the extra N^1 -Me excretion following a test dose of tryptophan (12, 13).

The substitution of corn products for wheat products in the diets of human subjects has led to decreased levels of N^1 -Me excretion ((15); Table V). In the earlier experiments the average N^1 -Me excretion by two subjects on the wheat diet which provided about 500 mg. of tryptophan was 1.1 and 2.0 mg., whereas, on the corn diet, which supplied about 380 mg. of tryptophan, the average excretion was lowered to 0.6 and 1.0 mg., respectively (15). In the present experiments, the replacement of wheat by a larger amount of corn decreased the tryptophan content of the diet by only 40 to 50 mg. The average N^1 -Me excretion by Subjects 21 and 22 changed from 2.1 and 2.6 mg. on the wheat diet to 1.4 and 1.7 mg., respectively, on the corn diet (Table V and the text). Since the addition of 500 mg. of L-tryptophan per day to the wheat diet of Subject 20 increased the N^1 -Me excretion from a basal average of 1.4 mg. to only 2.0 mg. per day⁶ (Table III), and the removal of 315 mg. of supplementary L-tryptophan from the diet of Subject 28 decreased the N^1 -Me excretion by about 1 mg. per day (Table IV), it is possible that the decrease in N^1 -Me excretion on the corn diet may be due in part to some other factor in addition to the decreased tryptophan intake (16).

Increasing the level of casein in the diet of the rat has been shown to lead either to no increase in excretion of N^1 -Me or to a smaller increase than would have been expected if free tryptophan were given in amounts equivalent to that present in the extra casein (8, 12, 20, 23). In the present experiments (Table IV) no change in excretion of nicotinic acid com-

⁶ Other experiments in this laboratory with small amounts of added tryptophan have also led to similar small increases in N^1 -Me excretion.

pounds was found after adding 30 gm. of casein per day to the diet, although the equivalent 315 mg. of tryptophan would have been sufficient to increase the excretion of nicotinic acid compounds.⁶ Possible reasons for the difference in conversion to nicotinic acid of "extra" tryptophan and of that present in protein have been discussed (8, 15).

SUMMARY

An amino acid imbalance (tryptophan-deficient) was created in the diets of four human subjects by the addition of 20 or 30 gm. of gelatin or 20 gm. of glycine daily for 10 to 12 days to diets low in protein and nicotinic acid. These additions led to changes in tryptophan excretion but had no significant effect upon the excretion of *N*¹-methylnicotinamide, nicotinic acid, "quinolinic acid," or 4-pyridoxic acid.

The addition to a similar basal diet of 30 gm. of vitamin-free casein or of 30 gm. of gelatin plus the amount of L-tryptophan equivalent to that present in the casein led to an increased excretion of tryptophan but had very little effect upon the excretion of nicotinic acid metabolites.

Replacement of part of the wheat products in a diet by corn products, which supplied 40 to 50 mg. less tryptophan per day than did the wheat, led to a decrease in excretion of tryptophan, *N*¹-methylnicotinamide, nicotinic acid, and "quinolinic acid," and an increase in excretion of 4-pyridoxic acid.

The author wishes to thank the following members of the Nutrition Research staff for their cooperation in these studies: Dr. Grace A. Goldsmith, Dr. Roy E. Butler, Dr. Frank Lossy, and Dr. George Jacobson for clinical assistance, Janis Gibbens and Jessica T. McCall for planning and supervising the diets, and Antoinette Dingraudo, Janice Loeb, Esther DiLeo, and Carol Haas for their technical assistance.

BIBLIOGRAPHY

1. Krehl, W. A., Teply, L. J., and Elvehjem, C. A., *Science*, **101**, 283 (1945).
2. Krehl, W. A., Teply, L. J., Sarma, P. S., and Elvehjem, C. A., *Science*, **101**, 489 (1945).
3. Powick, W. C., Ellis, N. R., and Dale, C. N., *J. Animal Sci.*, **7**, 228 (1948).
4. Spector, H., and Mitchell, H. H., *J. Biol. Chem.*, **165**, 37 (1946).
5. Groschke, A. C., and Briggs, G. M., *J. Biol. Chem.*, **165**, 739 (1946).
6. Singal, S. A., Sydenstricker, V. P., and Littlejohn, J. M., *J. Biol. Chem.*, **171**, 203 (1947).
7. Hanks, L. V., Henderson, L. M., Brickson, W. L., and Elvehjem, C. A., *J. Biol. Chem.*, **174**, 873 (1948).
8. Hundley, J. M., *J. Nutr.*, **34**, 253 (1947).
9. Groschke, A. C., Anderson, J. O., and Briggs, G. M., *Proc. Soc. Exp. Biol. and Med.*, **68**, 564 (1948).

10. Krehl, W. A., Sarma, P. S., and Elvehjem, C. A., *J. Biol. Chem.*, **162**, 403 (1946).
11. Krehl, W. A., Henderson, L. M., de la Huerga, J., and Elvehjem, C. A., *J. Biol. Chem.*, **166**, 531 (1946).
12. Schweigert, B. S., and Pearson, P. B., *J. Biol. Chem.*, **172**, 485 (1948).
13. Rosen, F., and Perlzweig, W. A., *J. Biol. Chem.*, **177**, 163 (1949).
14. Sarett, H. P., and Goldsmith, G. A., *J. Biol. Chem.*, **167**, 293 (1947).
15. Sarett, H. P., and Goldsmith, G. A., *J. Biol. Chem.*, **177**, 461 (1949).
16. Woolley, D. W., *J. Biol. Chem.*, **163**, 773 (1946).
17. Kuiken, K. A., Lyman, C. M., and Hale, F., *J. Biol. Chem.*, **171**, 551 (1947).
18. Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **167**, 157 (1947).
19. Sarett, H. P., *Proc. Soc. Exp. Biol. and Med.*, **69**, 494 (1948).
20. Singal, S. A., Briggs, A. P., Sydenstricker, V. P., and Littlejohn, J. M., *J. Biol. Chem.*, **166**, 573 (1946).
21. Henderson, L. M., *J. Biol. Chem.*, **178**, 1005 (1949).
22. Conger, T. W., and Elvehjem, C. A., *J. Biol. Chem.*, **138**, 555 (1941).
23. Bell, G. H., Scheer, B. T., and Deuel, H. J., Jr., *J. Nutr.*, **35**, 239 (1948).

THE EFFECT OF B VITAMINS UPON THE METABOLISM OF DL-TRYPTOPHAN IN MAN*

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The effect of B vitamin deficiencies upon the metabolism of tryptophan has been studied in rats, dogs, and mice by measurement of the excretion of xanthurenic acid, kynurenine, kynurenic acid, or of nicotinic acid and N^1 -methylnicotinamide (N^1 -Me) (1-11). Pyridoxine-deficient animals have been found to excrete more xanthurenic acid and less N^1 -Me after tryptophan administration than did control animals (1-9). Thiamine or riboflavin deficiency in the rat decreased the conversion of added tryptophan to N^1 -Me and changed the proportions in which xanthurenic acid, kynurenine, and kynurenic acid appear in the urine after tryptophan administration (10, 11). Severe caloric restriction of a complete diet also impaired the ability of the rat to convert tryptophan to nicotinic acid compounds (12).

In previous studies on the metabolism of tryptophan and its conversion to nicotinic acid compounds by man, most of the subjects were maintained on diets low in protein and B vitamins (13-16). The present experiments show that the addition of extra B vitamins to these diets had no effect upon the conversion of supplementary DL-tryptophan to nicotinic acid, "quinolinic acid," and N^1 -Me, but markedly decreased the urinary excretion of an ether-soluble tryptophan-like metabolite. Extra nicotinamide in the diet also had no effect upon the conversion of tryptophan to nicotinic acid compounds as evidenced by measurement of these metabolites in the urine.

EXPERIMENTAL

The subjects were ward patients similar to those employed in previous studies (13-16) and were maintained on the wheat¹ diet (14, 16). The

* A preliminary report was presented before the American Institute of Nutrition at Detroit, Michigan, April, 1949 (*Federation Proc.*, **8**, 394 (1949)). This work was supported by grants from the Nutrition Foundation, Inc., the Williams-Waterman Fund of the Research Corporation, and the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

¹ The unenriched wheat products were generously supplied by General Mills, Inc., through the courtesy of Dr. F. C. Hildebrand and Dr. H. S. Faulkner.

recorded food intakes are given in Tables I to III. Tryptophan² and vitamin supplements were divided between dinner and supper.

Urines were collected and pooled as previously described (14, 16). Creatinine and *N*¹-Me were measured by chemical methods (16) and nicotinic acid and tryptophan by microbiological methods (14). The estimation of "quinolinic acid" from nicotinic acid values obtained after acid autoclaving of the urine and the measurement of an ether-soluble tryptophan metabolite have been described (14, 16). In one of the experiments, tryptophan analyses were also performed by a chemical method (17), by which L- and D-tryptophan, the ether-extractable tryptophan-like compound, and other similar indole compounds, which may be present, are measured.

Xanthurenic acid was determined in one of the experiments by the method of Porter, Clark, and Silber (5, 11). Basal urines and those obtained after administration of DL-tryptophan do not give the typical color reaction of xanthurenic acid, and the values can only be considered as approximations.³

Results

Two female subjects (Subject 18, L. B., age 43, weight 64 kilos; Subject 19, S. D., age 56, weight 60 kilos) were maintained on the wheat diet for 16 days with no added nicotinamide and for the following 18 days with 50 mg. of nicotinamide per day. During the last 8 days of each of these periods, the subjects received a supplement of 5 gm. of DL-tryptophan per day. The excretion of nicotinic acid, "quinolinic acid" (as partially measured by the increase in nicotinic acid value after acid autoclaving), *N*¹-Me, and tryptophan (before and after ether extraction) by both subjects is shown in Table I. The higher basal levels of excretion of nicotinic acid compounds by Subject 18 were presumably due to the inclusion of one glass of milk per day in the diet.

The "free" nicotinic acid excretion was not markedly affected by the addition of 5 gm. of tryptophan or of 50 mg. of nicotinamide to the diet (Table I). The "quinolinic acid" excretion (obtained from nicotinic acid values before and after acid autoclaving) was not affected by nicotinamide administration but was increased after tryptophan administration (Table I) (14, 16). The presence of added nicotinamide in the diet had no effect upon the extra "quinolinic acid" excretion after tryptophan supplementation.

*N*¹-Me excretion by both subjects was increased by about 11 to 13 mg.

² The DL-tryptophan used in these studies was generously furnished by the Winthrop-Stearns Chemical Company, Inc., through the courtesy of Mr. Kenneth Smoot.

³ Porter, C. C., personal communication.

during the first test period with tryptophan (Table I). After a new base-line of N^1 -Me excretion for the nicotinamide period was obtained (Days 17 to 26), the DL-tryptophan supplement again increased the N^1 -Me excretion by about 11 to 17 mg. This suggests that extra tryptophan is converted to nicotinic acid independently of the level of nicotinic acid in the diet. This agrees with the earlier observation in which a subject on a good

TABLE I

Effect of Added Nicotinamide in Diet upon Daily Excretion of Related Metabolites Following Administration of Tryptophan

Diet	Day No.	Nicotinic acid				N^1 -Me		Tryptophan			
		Before acid autoclaving		After acid autoclaving				Before ether extraction		After ether extraction	
		Sub- ject 18	Sub- ject 19	Sub- ject 18	Sub- ject 19	Sub- ject 18	Sub- ject 19	Sub- ject 18	Sub- ject 19	Sub- ject 18	Sub- ject 19
		mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Wheat*	1-2	0.8	0.5	2.3	1.2	4.1	2.0	11	15	11	15
	3-4	0.7	0.7	2.3	1.7	4.2	2.7	11	20	10	19
	5-6	0.6	0.8	2.2	1.5	5.2	2.5	11	17	10	16
	7-8	0.6	0.7	2.1	1.4	4.2	2.6	10	18	9	16
Wheat + 5 gm. DL-tryptophan per day	9-10	0.8	0.6	4.4	3.0	10	8	96	100	43	54
	11-12	0.7	0.9	4.5	3.3	15	13	105	118	45	55
	13-14	0.7	0.8	4.2	3.4	18	16	99	121	43	61
	15-16	0.8		4.4		15		101		51	
Wheat + 50 mg. nicotinamide per day	17-18	0.6	0.7	2.0	1.5	17	16	15	15	10	13
	19-20		0.7		1.3		13		11		10
	21-22	0.6	0.8	2.0	1.4	15	12	9	10	8	9
	23-24	0.5	0.7	2.0	1.4	15	13	9	11	8	9
	25-26	0.6		1.9		16		9		8	
Wheat + 50 mg. nicotinamide + 5 gm. DL-trypto- phan per day	27-28	0.7	0.9	4.0	3.0	22	26	98	115	49	62
	29-30	0.7	0.9	4.1	3.2	27	27	127	115	52	66
	31-32	0.8	0.8	4.0	3.1	25	32	120	126	51	67
	33-34	0.7	1.0	4.1	3.0	28	30	116	107	63	54

* Subject 18 received one glass of milk per day. Average intake, Subject 18, 1740 calories, 36 gm. of protein, 4.4 mg. of nicotinic acid. Subject 19, 2050 calories, 34 gm. of protein, 5.1 mg. of nicotinic acid.

normal diet excreted as much extra N^1 -Me after tryptophan administration as did those subjects on the poor wheat or corn diets (14). Spector has shown that rats receiving nicotinic acid excrete more N^1 -Me after dosage with tryptophan than do rats which received no nicotinic acid (10).

The extra excretion of tryptophan and of the ether-extractable tryptophan-like compound after the administration of DL-tryptophan was also not affected by the nicotinamide supplement in the diet (Table I).

In testing the effect of other B vitamins upon the metabolism of DL-tryptophan the plan was similar to that of the above experiments. A male subject (No. 15, W. F., age 29, weight 80 kilos) received the wheat diet without vitamin supplementation for 20 days, and the same diet with vitamin B supplementation for 22 days. The vitamin B mixture supplied 2

TABLE II
Effect of Supplementary B Vitamins upon Daily Excretion of Related Metabolites Following Administration of Tryptophan to Subject 15

Diet	Day No.	Nicotinic acid		N ¹ -Me	Tryptophan	
		Before acid autoclaving	After acid autoclaving		Before ether extraction	After ether extraction
		mg.	mg.	mg.	mg.	mg.
Wheat (2600 calories, 42 gm. protein, 6.1 mg. nicotinic acid)	1- 2	0.6	2.0	2.3	23	22
	3- 4	0.6	1.7	2.1	20	19
	5- 6	0.5	1.6	2.9	20	20
	7- 8	0.6	1.9	2.5	23	22
Wheat + 5 gm. DL-tryptophan per day	9-10	0.9	3.9	10	117	66
	11-12	0.9	4.8	15	132	70
	13-14	0.8	5.0	16	145	59
	15-16	0.7	4.5	16	145	71
	17-18	0.8	5.0	16	151	73
	19-20	0.7	5.6	19	168	80
	21-22	0.7	3.0	10	25	20
Wheat + vitamin B mixture*	23-24	0.6	2.0	6	18	16
	25-26	0.7	2.1	4.3	21	20
	27-28	0.6	1.8	3.9	22	19
	29-30	0.5	1.8	3.5	22	22
	31-32	0.9	5.3	12	107	67
Wheat + vitamin B mixture* + 5 gm. DL-tryptophan per day	33-34	0.9	4.6	17	114	74
	35-36	0.8	4.3	17	114	68
	37-38	1.0	4.5	18	116	79
	39-40	0.8	4.9	13	95	64
	41-42	0.9	4.6	15	119	82

* The vitamin B mixture supplied 2 mg. of thiamine hydrochloride, 2 mg. of riboflavin, 2.5 mg. of folic acid, 5 mg. of calcium pantothenate, and 5 mg. of pyridoxine hydrochloride per day.

mg. of thiamine hydrochloride, 2 mg. of riboflavin, 2.5 mg. of folic acid, 5 mg. of calcium pantothenate, and 5 mg. of pyridoxine hydrochloride per day. During the last 12 days of each of the above periods, a daily supplement of 5 gm. of DL-tryptophan was also added (Table II). The increase in excretion of nicotinic acid, "quinolinic acid," and N¹-Me after tryptophan administration was similar in the presence or absence of the additional B vitamins in the diet (Table II).

Tryptophan excretion, as measured *after* ether extraction of the urine, was also unaffected by the vitamin B supplement, but the ether-removable tryptophan-like compound which is excreted after administration of DL-tryptophan (measured by the difference between tryptophan values before and after ether extraction) was decreased from an average of 72 mg. per day on the unsupplemented diet to 38 mg. per day on the diet with the

TABLE III

Effect of Supplementary B Vitamins upon Daily Excretion of Related Metabolites Following Administration of Tryptophan to Subject 24

Diet	Day No.	Nicotinic acid		N ^L -Me	Tryptophan			Xanthu- renic acid
		Before acid auto- claving	After acid auto- claving		Before ether extrac- tion	After ether extrac- tion	Chemical analysis	
		mg.	mg.	mg.	mg.	mg.	mg.	mg.
Wheat (1930 calo- ries, 33 gm. pro- tein, 5.0 mg. nicotinic acid)	1- 2	0.9	1.7	3.2	10	10		14
	3- 4	1.2	2.0	3.5	11	11		
	5- 6	0.7	2.1	2.7	12	11		
	7- 8	0.8	1.5	1.7	7	7		
	9-10	0.8	1.4	1.8	8	7		11
Wheat + 5 gm. DL-tryptophan per day	11-12	0.6	2.0	3	95	68	1320	52
	13-14	0.7	2.5	8	105	70	1500	49
	15-16	0.6	2.7	9	107	78	1500	
	17-18	0.9	2.8	12	116	69	1550	
	19-20	0.8	2.7	14	112	64	1400	46
Wheat + vitamin B mixture*	21-22	0.9	1.9	9	18	16		
	23-24							
	25-26	0.6	1.0	3	9	7		
	27-28	0.6	1.2	2.7	10	9		
	29-30	0.6	1.1	2.6	7	7		14
Wheat + vitamin B mixture* + 5 gm. DL-trypto- phan per day	31-32	0.7	2.4	7	83	62	1370	52
	33-34	0.9	2.5	14	105	74	1390	
	35-36	1.0	3.0	15	106	84	1350	
	37-38	0.9	2.8	13	112	97	1260	
	39-40	1.1	2.9	14	116	108	1220	56

* The vitamin B mixture supplied 5 mg. each of thiamine hydrochloride, riboflavin, folic acid, calcium pantothenate, and pyridoxine hydrochloride per day.

added B vitamins. The level of this metabolite in the urine appears to be significantly lowered by the presence of the extra B vitamins in the diet. This is also evident from the results with Subject 24 (H. W., male, age 53, weight 67 kilos), as shown in Table III.

The experiment on Subject 24 was carried out in the same manner as with Subject 15, except that the vitamin B mixture was increased to 5 mg. each of thiamine hydrochloride, riboflavin, folic acid, calcium pantothen-

ate, and pyridoxine hydrochloride. The addition of these vitamins to the diet did not affect the conversion of extra tryptophan to nicotinic acid compounds, but did change the excretion of tryptophan compounds. Tryptophan values, as measured *after* ether extraction of the urine, were increased more when the extra B vitamins were administered with the tryptophan than when the tryptophan was the sole supplement. The excretion of the ether-extractable tryptophan-like compound was decreased from an average of 37 mg. per day on the unsupplemented diet to 19 mg. per day on the diet containing the added B vitamins (Table III). During the last 2 days of the tryptophan and vitamin test period (Days 39 and 40) only 8 mg. per day was found. One or more of the supplementary B vitamins may be involved in the further breakdown of this metabolite of DL-tryptophan, or may inhibit the formation of this substance.

The values for total tryptophan excretion, as measured chemically, in the urines obtained after tryptophan administration differed only slightly when the vitamin B supplement was absent or present in the diet (Table III). These values represent mainly D-tryptophan excretion and show that only part (1.2 to 1.6 gm.) of the 2.5 gm. of D-tryptophan present in the 5 gm. of DL-tryptophan was excreted in the urine (18).

Xanthurenic acid analyses were carried out on some of the urines, and, although the values are only approximate (see "Experimental"), they are presented in Table III. The addition of 5 gm. of DL-tryptophan to the diet increased the xanthurenic acid excretion by about 35 to 40 mg. The extra B vitamins had no effect on xanthurenic acid excretion, either with the basal diet or after tryptophan administration.

DISCUSSION

Although tryptophan is an essential amino acid, its partial conversion to nicotinic acid compounds by man is not prevented or inhibited when a large excess of nicotinamide is available. Previous data suggest that the per cent of tryptophan converted to nicotinic acid compounds also remains the same when various amounts of tryptophan are added to the diet. The extra *N*¹-Me which appeared in the urine after feeding supplementary tryptophan was proportional to the level of extra tryptophan (14).

The inhibition of growth of animals by diets which are relatively low in tryptophan in comparison with the other amino acids can be overcome by feeding extra tryptophan or nicotinic acid (6, 7, 19, 20). It is possible that the amino acid imbalance which limits growth creates a need for more nicotinic acid than is usually required. The rat grows well on a balanced protein diet devoid of nicotinic acid (20, 21). In the light of the present experiments in man, it is doubtful whether nicotinic acid exerts its protective rôle in amino acid imbalance by "sparing" the tryptophan present in the diet (19, 20).

The metabolism of DL-tryptophan in man results in the excretion of a substance which can be readily extracted from the acidified urine with ether and can replace L-tryptophan for growth of *Lactobacillus arabinosus* (14). Recent experiments (22) indicate that D-tryptophan rather than the L isomer is normally the main precursor of this metabolite. The addition of a mixture of thiamine, riboflavin, pantothenic acid, pyridoxine, and folic acid to the diet significantly decreased the amount of this metabolite which was excreted. The findings suggest that one or more of these B vitamins may be involved in the further breakdown of this metabolite, or may inhibit the formation of this substance. The addition of the above B vitamins had no effect upon the conversion of tryptophan to nicotinic acid compounds.

SUMMARY

The addition of 50 mg. of nicotinamide per day to a diet low in protein and B vitamins had no effect upon the metabolism of extra DL-tryptophan as evidenced by the increase in excretion of *N*¹-methylnicotinamide, "quinolinic acid," tryptophan, and of an ether-extractable tryptophan-like compound. The findings indicate that the presence of a high level of nicotinamide in the diet does not inhibit the conversion of tryptophan to nicotinic acid.

Supplementation of the same diet with thiamine, riboflavin, pyridoxine, folic acid, and pantothenic acid had no effect upon the extra excretion of nicotinic acid compounds or of xanthurenic acid after DL-tryptophan administration, but resulted in a marked decrease in the extra excretion of the ether-extractable tryptophan-like compound.

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BIBLIOGRAPHY

1. Lepkovsky, S., Roboz, E., and Haagen-Smit, A. J., *J. Biol. Chem.*, **149**, 195 (1943).
2. Axelrod, H. E., Morgan, A. F., and Lepkovsky, S., *J. Biol. Chem.*, **160**, 155 (1945).
3. Reid, D. F., Lepkovsky, S., Bonner, D., and Tatum, E. L., *J. Biol. Chem.*, **155**, 299 (1944).
4. Miller, E. C., and Baumann, C. A., *J. Biol. Chem.*, **157**, 551 (1945).
5. Porter, C. C., Clark, I., and Silber, R. H., *J. Biol. Chem.*, **167**, 573 (1947).
6. Rosen, F., Huff, J. W., and Perlzweig, W. A., *J. Nutr.*, **33**, 561 (1947).
7. Schweigert, B. S., and Pearson, P. B., *J. Biol. Chem.*, **168**, 555 (1947).

8. Bell, G. H., Scheer, B. T., and Deuel, H. J., Jr., *J. Nutr.*, **35**, 239 (1948).
9. Ling, C.-T., Hegsted, D. M., and Stare, F. J., *J. Biol. Chem.*, **174**, 803 (1948).
10. Spector, H., *J. Biol. Chem.*, **173**, 659 (1948).
11. Porter, C. C., Clark, I., and Silber, R. H., *Arch. Biochem.*, **18**, 339 (1948).
12. Junqueira, P. B., and Schweigert, B. S., *J. Biol. Chem.*, **175**, 535 (1948).
13. Sarett, H. P., and Goldsmith, G. A., *J. Biol. Chem.*, **167**, 293 (1947).
14. Sarett, H. P., and Goldsmith, G. A., *J. Biol. Chem.*, **177**, 461 (1949).
15. Sarett, H. P., *Proc. Soc. Exp. Biol. and Med.*, **69**, 494 (1948).
16. Sarett, H. P., *J. Biol. Chem.*, **182**, 659 (1950).
17. Spies, J. R., and Chambers, D. C., *Anal. Chem.*, **20**, 30 (1948).
18. Perlzweig, W. A., Rosen, F., Levitas, N., and Robinson, J., *J. Biol. Chem.*, **167**, 511 (1947).
19. Krehl, W. A., de la Huerga, J., and Elvehjem, C. A., *J. Biol. Chem.*, **164**, 551 (1946).
20. Hundley, J. M., *J. Nutr.*, **34**, 253 (1947).
21. Dann, W. J., *J. Biol. Chem.*, **141**, 803 (1941).
22. Sarett, H. P., and Goldsmith, G. A., *J. Biol. Chem.*, **182**, 679 (1950).

METABOLISM OF L- AND DL-TRYPTOPHAN IN NORMAL MAN AND IN PELLAGRINS*

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Recent experiments have shown that the administration of L- or DL-tryptophan to man leads to an increased excretion of nicotinic acid compounds, mainly as *N*¹-methylnicotinamide (*N*¹-Me) (1-4) and partially as a compound which can be converted to nicotinic acid by autoclaving the urine in acid solution (3, 4). The latter substance is probably identical with quinolinic acid, which has been isolated from the urine of rats receiving DL-tryptophan (5). In human subjects who have taken DL-tryptophan, the excretion of tryptophan and of an ether-extractable tryptophan-like substance is also markedly increased (3, 4).

In the present experiments, the excretion of these nicotinic acid and tryptophan compounds has been measured after feeding L- or DL-tryptophan to normal control subjects and to pellagrins. In the control subjects, the ether-extractable tryptophan-like substance did not appear in the urine following dosage with L-tryptophan, but did after DL-tryptophan. The excretion in pellagrins, following L-tryptophan therapy, showed marked differences from the normal in this and other respects.

EXPERIMENTAL

The control subjects included one ward patient (Subject 23), similar to those employed in previous studies (1, 3, 4, 6), and one member of the staff (Subject 27). The pellagrins were patients in the Negro wards at the Charity Hospital of Louisiana and received no therapy prior to these studies. Two subjects with chronic nephritis and uremia were also studied, since the experiment on one of the pellagrins (Subject 16) was started while he had a marked azotemia, and it was considered that some of the excretory findings might have been due to the azotemia.

The pellagrins and one of the control subjects (No. 23) were maintained

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on the wheat or corn diet¹ previously described (3, 6). The other control subject (No. 27) and the two uremic subjects were kept on more conventional diets, which were controlled as rigorously as were the corn and wheat diets. The average dietary intakes which are given in Tables I to VII do not include the test supplements. All supplements to the diets were divided between dinner and supper. Subject 27 continued his regular work during the experiment, whereas the others were maintained in the hospital wards or in a special metabolism ward.

24 hour urines were collected as previously described (3, 4, 6), and in some of the experiments were analyzed as such, whereas in others they were pooled in 48 hour periods. Creatinine, nitrogen, *N*¹-Me, and 4-pyridoxic acid were measured by chemical methods, and tryptophan and nicotinic acid by microbiological methods (3, 6). Creatinine values are not reported, but were obtained in all urines to check the completeness of collection.

Quinolinic acid, which is decarboxylated to nicotinic acid by autoclaving in acid solution, and an ether-soluble tryptophan-like compound which can replace L-tryptophan for growth of *Lactobacillus arabinosus*, have recently been found to be metabolites of tryptophan (3-6) and were also measured (3, 6).

Total tryptophan compounds were measured in four of the experiments by a chemical method (4, 7). Only approximate values can be obtained in urines low in tryptophan by this method.

Xanthurenic acid analyses were carried out by the method of Porter, Clark, and Silber (8). The method is not sensitive enough to give accurate values in control urines, but can be used to measure xanthurenic acid excretion after administration of L-tryptophan. DL-Tryptophan supplements lead to the excretion of metabolites which interfere with the test² (4).

Results

The excretion of nicotinic acid and tryptophan metabolites after administration of L- or DL-tryptophan³ to a control subject (No. 23, S. D., female, age 56, weight 60 kilos) maintained on a wheat diet is shown in Table I. Since the D form of tryptophan is not utilized for maintenance of nitrogen balance by man,⁴ the addition of 2.5 gm. of L-tryptophan was compared

¹ The unfortified corn products used in these diets were generously provided by the Quaker Oats Company, through the courtesy of Dr. F. N. Peters, and the unenriched wheat products by General Mills, Inc., through the courtesy of Dr. F. C. Hildebrand and Dr. H. S. Faulkner.

² Porter, C. C., personal communication.

³ L-Tryptophan was generously supplied by Dr. C. Robertson of the Van Camp Laboratories. The DL-tryptophan was generously furnished by the Winthrop-Stearns Chemical Company, Inc., through the courtesy of Mr. Kenneth Smoot.

⁴ Rose, W. C., personal communication.

with 5 gm. of DL-tryptophan. Each of these tryptophan supplements led to somewhat comparable increases in excretion of N^1 -Me, nicotinic acid and "quinolinic acid." The increase in nicotinic acid values found after acid autoclaving represents part of the "quinolinic acid" which is decarboxylated (3-6). Under the conditions employed in these experiments about one-fifth of the "quinolinic acid" is converted to nicotinic acid (3, 4, 6).

TABLE I

Effect of Administration of L- and DL-Tryptophan upon Daily Excretion of Related Compounds by Subject 23

Diet	Day No.	Nitrogen	Nicotinic acid		N^1 -Me	Tryptophan			4-Pyridoxic acid
			Before acid autoclaving	After acid autoclaving		Before ether extraction	After ether extraction	Chemical analysis	
		gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Wheat (2050 calories, 33 gm. protein, 5.2 mg. nicotinic acid)	1- 2	5.5	0.5	1.1	2.5	12.1	11.7		1.3
	3- 4	4.0	0.4	0.9	2.3	10.1	10.3	6	1.0
	5- 6	4.7	0.4	1.2	3.2	12.4	12.1	12	1.2
	7- 8	3.8	0.5	1.1	2.6	11.1	10.5		1.3
	9-10	4.1	0.4	1.2	2.8	12.0	11.2		1.3
Wheat + 2.5 gm. L-tryptophan per day	11-12	4.3	0.5	2.1	7	25	25		1.2
	13-14	4.4	0.6	2.2	12	27	26	23	1.3
	15-16	4.2	0.7	2.3	13	27	25		1.2
	17	5.2	0.9	2.7	17	30	28	19	1.2
Wheat	19-20	4.3	0.6	1.3	7.4	10.6	10.3		1.3
	21-22	4.3	0.6	1.1	3.4	9.9	9.2	8	1.2
	23-24	4.7	0.5	0.9	2.4	11.7	8.0	7	1.4
	25-26	4.0	0.4	0.8	2.5	8.5	7.8		1.0
	27-28	4.3	0.4	0.9	2.1	12.6	10.9		1.4
Wheat + 5 gm. DL-tryptophan per day	29-30	4.5	0.5	1.9	8	87	36	1500	1.8
	31-32	5.1	0.6	2.0	17	105	50	1700	2.7
	33-34	5.2	0.7	2.2	20	110	54	1650	2.0
	35-36	5.5	1.0	3.7	24	151	72	1850	4.5

Tryptophan excretion (measured after ether extraction) was much higher after administration of 5 gm. of DL-tryptophan (average 53 mg.) than after 2.5 gm. of L-tryptophan (average 26 mg.). The D form of the amino acid is not measured by assay with *L. arabinosus* (9).

The ether-extractable tryptophan-like substance, which has been found in the urine of several subjects who received DL-tryptophan (3, 4), was greatly increased after administration of 5 gm. of DL-tryptophan to Subject 23 (average 60 mg. per day) but not at all after 2.5 gm. of L-tryptophan (Table I). It seems, therefore, that the ether-extractable tryptophan-like compound is mainly a metabolite of D-tryptophan and not of L-tryptophan. Total tryptophan analyses by a chemical method gave values of the order

of magnitude of those determined microbiologically in the basal urines and in those obtained after administration of L-tryptophan. When 5 gm. of DL-tryptophan were given, 1.5 to 1.85 gm. of total tryptophan were found by the chemical method (Table I; (2)). This did not account for all of the D-tryptophan which was administered.

Ingestion of large amounts of DL-tryptophan has been shown to interfere with the analysis of 4-pyridoxic acid in urine (3). The results in Table I

TABLE II
Effect of Administration of L- and DL-Tryptophan upon Daily Excretion of Related Compounds by Subject 27

Diet	Day No.	Nitro- gen	Nicotinic acid		N ¹ -Me	Tryptophan			4-Pyri- doxic acid	Xanthu- renic acid
			Be- fore acid auto- clav- ing	After acid auto- clav- ing		Be- fore ether extrac- tion	After ether extrac- tion	Chem- ical analy- sis		
		gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Control (2200 calories, 95 gm. protein, 17 mg. nicotinic acid)	1- 2	15.4	0.7	1.8	7	23	22		1.8	24
	3- 4	14.2	0.8	1.6	8	22	21		1.9	27
Control + 2.5 gm. L- tryptophan per day	5	16.5	0.8	2.5	8	37	34	17	2.0	29
	6	15.5	0.9	2.7	12	35	31	13	1.5	24
	7	14.5	0.8	2.7	9	33	31	19	1.8	28
Control + 5 gm. L- tryptophan per day	8	17.4	0.8	3.7	16	52	47	27	1.7	36
	9	17.7	1.1	3.5	17	53	50	36	2.3	22
	10	16.1	1.0	3.8	20	55	52	40	1.7	21
Control	11-12	16.3	0.8	1.8	11	25	24	11	1.9	14
	13-14	14.6	0.7	1.5	8	23	23	11	2.0	
Control + 5 gm. DL- tryptophan per day	15	15.5	0.9	2.1	10	75	54	1350	2.7	
	16	14.6	0.8	2.2	13	84	61	1400	1.8	(46)*
	17	16.7	0.8	2.2	15	81	60	1400	2.8	
Control + 10 gm. DL- tryptophan per day	18	16.7	0.9	2.9	19	155	90	3300	2.7	(91)*
	19	14.1	0.9	2.6	19	158	87	3100	2.6	
	20	15.9	1.0	2.9	24	171	95	3050	3.3	(92)*

* Approximate; see the text.

show that the administration of L-tryptophan had no effect upon the level of excretion of 4-pyridoxic acid. After DL-tryptophan administration the high values were due to the tryptophan metabolites which interfere with the method.

Another subject (No. 27, G. J., male, age 24, weight 78 kilos) was maintained on a more adequate diet in a similar study of tryptophan metabolism (Table II). After 2.5 or 5 gm. of L-tryptophan per day the excretion of "quinolinic acid" was slightly higher and the excretion of N¹-Me slightly lower than after the corresponding amounts (5 or 10 gm.) of DL-tryptophan

(Table II). In general, however, it appears that only the L isomer of tryptophan was effective as a nicotinic acid precursor (Tables I and II).

The excretion of tryptophan by Subject 27 after supplements of L- or DL-tryptophan (Table II) was similar to that found for Subject 23 in Table I. Administration of L-tryptophan led to smaller increases in excretion of tryptophan than did administration of DL-tryptophan. With 2.5 and 5 gm. of L-tryptophan daily, virtually no increase was observed in excretion of the ether-extractable component, whereas the average excretion after 5 and 10 gm. of DL-tryptophan amounted to 22 and 70 mg., respectively. The 22 mg. of this compound excreted after 5 gm. of DL-tryptophan by Subject 27 were much less than the 53 mg. excreted by Subject 23 (Table I). The improved basal diet may account, in part, for this difference between the two subjects, since it has been shown that extra B vitamins in the diet decreased the excretion of the ether-extractable tryptophan-like compound after administration of DL-tryptophan (4). Tryptophan analyses by a chemical method show that about 1.4 and 3.1 gm. of total tryptophan were accounted for after administration of 5 and 10 gm. of DL-tryptophan, respectively, to Subject 27.

The administration of L-tryptophan led to no significant change in excretion of xanthurenic acid (Table II). After DL-tryptophan administration the metabolites which were excreted interfered with the test and gave high values which were not a measure of xanthurenic acid.

The pattern of excretion of 4-pyridoxic acid was similar to that of Subject 23. The administration of L-tryptophan had no effect upon the level of excretion, whereas a false increase was obtained after DL-tryptophan (3).

A subject (No. 13, T. P., male, age 45, weight 47 kilos) with chronic pellagra, severe loss of weight, and amebiasis was maintained on the wheat diet, and received 5 gm. of L-tryptophan per day for 4 days. It was possible to obtain a control urine for only 1 day prior to this therapy. After the 5 day study the patient's amebiasis had to be treated and the tryptophan studies were stopped. Little clinical improvement could be noted after the 4 days of tryptophan administration, but the patient responded slowly to subsequent therapy with nicotinamide, riboflavin, and thiamine.

The excretory findings are shown in Table III. N^1 -Me excretion following L-tryptophan administration increased more slowly than in normal subjects, presumably because of the nicotinic acid deficiency of this subject. The urinary nicotinic acid values also increased slightly, while the values after autoclaving with acid showed the presence of much more "quinolinic acid" (12 to 20 mg.) than has been observed in normal subjects who have received comparable amounts of L- or DL-tryptophan (Tables I and II; (3, 4)).

Subject 13 excreted 250 to 300 mg. of tryptophan in the first 2 days

after receiving 5 gm. of L-tryptophan per day, and excreted very little of the ether-extractable tryptophan-like compound until the 3rd and 4th days of tryptophan dosage (Table III). Normal persons excreted only about 50 mg. of tryptophan and practically none of the ether-extractable compound when 5 gm. of L-tryptophan were administered daily. The L-tryptophan supplement also led to an increased excretion of xanthurenic acid (60 to 130 mg. per day) by the pellagrin, Subject 13. This was not found to be true in a normal subject (Table II).

Subject 25 (A. C., female, age 39, weight 50 kilos) appeared to be generally malnourished and showed signs of chronic pellagra: atrophy of the papillae of the tongue, resulting in red smooth patches, and pigmented symmetrical dermatitis on the forearms, wrists, lower legs, and neck. The

TABLE III
Effect of Administration of L-Tryptophan upon Daily Excretion of Related Compounds by Subject 13, with Pellagra

Diet	Day No.	Nitrogen	Nicotinic Acid		N ¹ -Me	Tryptophan		Xanthu- renic acid
			Before acid auto- claving	After acid auto- claving		Before ether extrac- tion	After ether extrac- tion	
		gm.	mg.	mg.	mg.	mg.	mg.	mg.
Wheat*	1	3.9	0.3	0.7	1.2	26	24	0
“ + 5 gm. L- tryptophan per day	2	4.2	0.5	6.5	2.8	265	255	60
	3	4.6	0.6	9.0	4.0	318	300	55
	4	5.3	0.9	12.5	4.9	117	90	80
	5	6.4	1.1	20.3	9.5	160	128	130

* Average intake, 930 calories, 15 gm. of protein, 2.1 mg. of nicotinic acid.

average excretion of nitrogen, tryptophan, and nicotinic acid compounds by Subject 25 during 17 days on the basal corn diet was low (Table IV) and there was no significant improvement in the clinical appearance of the patient at this time. L-Tryptophan therapy for 5 days resulted in a relatively prompt increase in excretion of N¹-Me, “quinolinic acid,” and of tryptophan (Table IV), and some improvement in the clinical findings. It is of interest to note that, in contrast to the controls, Subject 25 excreted a significant amount of the ether-extractable tryptophan-like substance (11 mg. average per day) after receiving L-tryptophan.

During a second 6 day basal period on the corn diet all of the excretion values fell to the low levels associated with this diet. Response to 10 gm. of DL-tryptophan per day was essentially normal as evidenced by the excretory findings (Table IV). Subject 25 continued to receive 10 gm. of DL-tryptophan per day for 22 days after urinary analyses were discontinued,

and at this time there was marked improvement in her clinical condition with almost complete disappearance of the dermatitis and regeneration of the lingual papillae.

Another pellagrin (Subject 16, G. F., male, age 71, weight 53 kilos), who was starved, dehydrated, and had azotemia at time of admission, showed a red, dry tongue, dry, pigmented, scaly skin on the arms and legs, and dark thickened patches over the elbows, knees, and feet. It was possible to obtain only one control urine on the wheat diet before therapy with L-tryp-

TABLE IV

Effect of Administration of L- and DL-Tryptophan upon Daily Excretion of Related Metabolites by Subject 25, with Pellagra

Diet	Day No.	Nitrogen	Nicotinic acid		N ¹ -Me	Tryptophan		
			Before acid auto-claving	After acid auto-claving		Before ether extraction	After ether extraction	Chemical analysis
		gm.	mg.	mg.	mg.	mg.	mg.	mg.
Corn*	1-17	3.0	0.3	0.7	0.8	5.3	4.3	
" + 5 gm. L-tryptophan per day	18	3.2	0.3	3.2	2	29	23	
	19	2.5	0.7	8.0	15	87	73	
	20	2.8	0.7	6.1	21	81	69	
	21	2.6	0.6	5.5	20	59	47	
	22†	2.7	0.7	4.9	26	60	51	
Corn	23-24	2.4	0.4	1.0	7	8	7	
	25-26	2.8	0.4	0.9	3	7	6	
	27-28	2.7	0.4	0.8	1	6	5	
Corn + 10 gm. DL-tryptophan per day	29	2.6	0.4	4.4	6	200	83	2250
	30	2.8	0.7	7.9	28	230	123	2400
	31	2.6	0.7	6.1	27	171	89	1800
	32	3.0	0.7	6.4	31	193	91	2100

* Average intake, 1570 calories, 26 gm. of protein, 4.0 mg. of nicotinic acid to the 10th day; 1700 calories, 30 gm. of protein, 4.3 mg. of nicotinic acid after the 10th day.

† Only 4 gm. of L-tryptophan given on 22nd day.

tophan was initiated (Table V). The extent of starvation and dehydration is shown by the elevated level of blood urea nitrogen and the large output of nitrogen, N¹-Me, and tryptophan on the control day (Table V). The urea nitrogen was 106 mg. per 100 ml. of blood at this time. Increased food intake and hydration led to a decrease in azotemia and a diminished loss of body nitrogen (Table V). The blood urea nitrogen decreased progressively from 81 mg. per 100 ml. on the 2nd day to 39 mg. per 100 ml. on the 5th day. On the 9th day 19 mg. of urea nitrogen per 100 ml. of blood were found.

On the 1st day of the basal diet Subject 16 excreted abnormally large

amounts of “quinolinic acid” and of the ether-extractable tryptophan-like compound (Table V). This had not been found in any of the controls or pellagrins, and it was considered that the state of dehydration and azotemia may have contributed to these findings (see below, Subjects 26 and 30). 5 gm. of L-tryptophan per day for 5 days gradually increased the excretion of *N*¹-Me by Subject 16, and the level of “quinolinic acid” became abnormally high (Table V). A significant amount of the ether-ex-

TABLE V
Effect of Administration of L- and DL-Tryptophan upon Daily Excretion of Related Compounds by Subject 16, with Pellagra

Diet	Day No.	Nitrogen	Nicotinic acid		<i>N</i> ¹ -Me	Tryptophan		Xanthu- renic acid
			Before acid auto- claving	After acid auto- claving		Before ether extrac- tion	After ether extrac- tion	
		gm.	mg.	mg.	mg.	mg.	mg.	mg.
Wheat*	1	25.2	0.9	14	12	40	29	23
“ + 5 gm. L- tryptophan per day	2	18.9	0.9	31	9	65	44	600
	3	16.8	1.1	53	14	66	43	450
	4	15.4	1.1	58	19	72	44	500
	5	12.8	0.9	51	19	60	39	300
	6	10.3	0.9	43	18	61	44	360
Wheat	7	9.6	1.2	30	23	25	21	70
	8	7.8	1.0	11	15	20	17	35
	9	9.8	1.2	7	10	25	24	30
	18	4.9	1.3	3	4	15	13	
	19	4.9	1.3	3	3	12	11	
Wheat + 10 gm. DL-tryptophan per day	20	5.1	0.6	6	5	241	40	
	23	5.0	0.6	9	10	125	76	
	24	5.2	0.5	7	9	142	79	
	25	6.1	0.8	8	11	125	52	
	31	4.5	0.6	8	11	195	140	

* Average intake, Days 1 to 9, 1300 calories, 15 gm. of protein, 2.5 mg. of nicotinic acid; Days 18 to 31, 2400 calories, 37 gm. of protein, 5.4 mg. of nicotinic acid.

tractable tryptophan component (average 22 mg. per day) was excreted in the urine after L-tryptophan administration, as has been noted in the other pellagrins. An unusually high level of xanthurenic acid excretion following administration of L-tryptophan was also found in Subject 16. Definite improvement in the condition of the tongue and of the skin on the legs was observed during the 5 day period.

After another 13 days on the wheat diet, during which the subject became well hydrated, the blood urea nitrogen and the urinary excretion dropped to normal levels (Table V). The administration of 10 gm. of

DL-tryptophan per day resulted in the excretion of relatively normal amounts of "quinolinic acid" and of tryptophan compounds, and a low output of N^1 -Me. Subject 16 improved greatly during the month on the wheat diet, with the tryptophan supplements.

In view of the findings in the pellagrin with azotemia (Table V), a subject without pellagra (Subject 26, E. L. B., female, age 43, weight 66 kilos), who had chronic nephritis and uremia, was studied. The level of blood urea nitrogen was followed at frequent intervals throughout and was found to average 55 mg. (49 to 61 mg.) per 100 ml. Subject 26 was maintained on a low salt diet for 5 days prior to administration of 10 gm. of DL-tryptophan per day. She was discharged from the hospital before it was possible to study the metabolism of L-tryptophan.

TABLE VI

Effect of Administration of DL-Tryptophan upon Daily Excretion of Related Compounds by Subject 26, with Nephritis

Diet	Day No.	Nitrogen	Nicotinic acid		N^1 -Me	Tryptophan	
			Before acid auto-claving	After acid auto-claving		Before ether extraction	After ether extraction
		gm.	mg.	mg.	mg.	mg.	mg.
Low salt control (1750 calories, 60 gm. protein, 11 mg. nicotinic acid)	1		0.3	1.5	2.0	23	19
	2	8.8	0.2	1.2	1.7	21	16
	3	8.8	0.3	1.8	1.7	20	19
	4	6.9	0.2	1.6	1.6	15	12
	5	7.2	0.3	1.7	2.3	19	16
Low salt control + 10 gm. DL-tryptophan per day	6	7.1	0.3	3	4	42	27
	7	8.3	0.4	18	14	85	44
	8	7.1	0.4	24	11	81	30
Low salt control	9	7.2	0.5	21	9	54	17

On the basal diet, the average free nicotinic acid excretion was less than 0.3 mg. per day, while the nicotinic acid and "quinolinic acid" excretion (obtained after acid autoclaving) averaged 1.6 mg. per day (Table VI). This total value (1.6 mg. per day) was in the normal range, but was over 5 times the free nicotinic acid value, whereas in most normal subjects this ratio has been found to be about 2 or 3 to 1 (Table I and II). The excretion of tryptophan compounds by Subject 26 showed the presence of about 3 mg. per day of the ether-extractable compound in the basal urines in contrast to the 0.5 to 1 mg. in the controls (Tables I and II; (3, 4)). These variations from normal findings are similar to, but much lower than, those observed in the pellagrin with azotemia (Table V).

After receiving 10 gm. of DL-tryptophan per day, Subject 26 excreted

an unusually large amount of "quinolinic acid" (Table VI); this was similar to the results with pellagrins, Subjects 13 and 16. The excretion of tryptophan compounds, however, was markedly lower than normal.

Table VII shows the results obtained with another patient with glomerulonephritis and uremia (Subject 30, F. D. B., male, age 68, weight 65 kilos). The food intake was quite variable, as is shown in the note below

TABLE VII

Effect of Administration of L- and DL-Tryptophan upon Daily Excretion of Related Compounds by Subject 30, with Nephritis

Diet	Day No.	Nitrogen	Nicotinic acid		N ¹ -Me	Tryptophan		
			Before acid auto-claving	After acid auto-claving		Before ether extraction	After ether extraction	Chemical analysis
		gm.	mg.	mg.	mg.	mg.	mg.	mg.
Control*	1	11.1	0.5	2.3	1.1	23	20	
	2	10.4	0.3	1.8	1.2	27	24	100
	3	11.2	0.4	1.6	1.2	27	23	
Control + 5 gm. L-tryptophan per day	4	11.2	0.9	3.4	1.8	78	69	150
	5	11.5	0.5	8.5	2.0	65	54	140
Control	6	10.3	0.6	9.0	1.2	32	27	
	7	8.2	0.6	4.4	1.3	31	29	
Control + 10 gm. DL-tryptophan per day	8	8.5	0.4	4.5	2.0	54	46	670
	9	10.3	0.6	12.8	3.0	78	68	1300
Control	10	8.7	0.6	14.0	2.7	56	44	
	11	8.2	0.7	8.6	2.4	47	40	320
	12	8.7	0.7	5.7	1.9	38	30	
Control + 5 gm. L-tryptophan per day	13	8.5	0.6	5.6	2.0	43	36	150
	14	8.5	0.6	9.3	2.5	41	34	110

* Average intake, Days 1 to 3, 2100 calories, 68 gm. of protein, 11.4 mg. of nicotinic acid; Days 4 to 5, 1740 calories, 53 gm. of protein, 10.1 mg. of nicotinic acid; Days 6 to 7, 500 calories, 11 gm. of protein, 1.7 mg. of nicotinic acid; Days 8 to 9, 1200 calories, 44 gm. of protein, 7.0 mg. of nicotinic acid; Days 10 to 12, 1670 calories, 48 gm. of protein, 5.9 mg. of nicotinic acid; Days 13 to 14, 1720 calories, 54 gm. of protein, 8.0 mg. of nicotinic acid.

Table VII. The blood urea nitrogen averaged 61 mg. (53 to 72 mg.) per 100 ml. The urinary excretion was similar to that in the other nephritic subject (No. 26). The excretion of "quinolinic acid" after administration of L- or DL-tryptophan was somewhat higher than in the controls. In Subjects 16, 26, and 30 with azotemia, who excreted abnormally large amounts of "quinolinic acid," the excretion of this compound has been

found to continue at a high level for a day or two after tryptophan administration was stopped.

Subject 30 also excreted a significant amount of the ether-extractable tryptophan-like substance after receiving L-tryptophan (Days 4, 5 and 13, 14). The L-tryptophan supplement on Days 13 and 14 led to a very small increase in tryptophan excretion as measured microbiologically. After receiving DL-tryptophan, the excretion of tryptophan, the ether-extractable tryptophan component, and of total tryptophan compounds (chemical analysis) by Subject 30 was much lower than normal. The tryptophan analyses by the chemical method gave values much higher than normal in the basal urines and in those obtained after L-tryptophan administration.

DISCUSSION

In normal subjects the administration of L- or DL-tryptophan led to an increase in the excretion of *N*¹-Me and of "quinolinic acid." After DL-tryptophan was given, there was an increase in excretion of L-tryptophan and of an ether-extractable compound which can replace L-tryptophan for the growth of *L. arabinosus*. In contrast, the administration of comparable amounts of L-tryptophan led to a smaller increase in excretion of L-tryptophan, with practically none of the ether-extractable compound. These results suggest that the latter metabolite is formed mainly from D-tryptophan in normal subjects.

Each of the three pellagrins in the present study differed from one another and varied from normal in the excretion of nicotinic acid and tryptophan compounds after receiving 5 gm. of L-tryptophan per day. The only consistent finding in all three subjects was the excretion of some of the ether-extractable tryptophan-like compound following L-tryptophan. This compound did not appear in the urines of control subjects after L-tryptophan, but was found in the urine of a subject with nephritis who received L-tryptophan.

The high levels of this ether-extractable tryptophan compound and of "quinolinic acid" in the control urine of the pellagrin, Subject 16 (Table V), may have been due in part to azotemia. Experiments on two subjects with chronic nephritis and uremia showed an unusually high excretion of these compounds in the basal urines, and of "quinolinic acid" after the tryptophan supplements.

SUMMARY

The urinary excretion of several nicotinic acid and tryptophan metabolites by two control subjects was compared after administration of L- and DL-tryptophan. Approximately twice as much DL-tryptophan in com-

parison with L-tryptophan was required to produce about the same increase in excretion of *N*¹-methylnicotinamide and "quinolinic acid." When 2.5 gm. of L-tryptophan were given as such, much less L-tryptophan appeared in the urine than when 5 gm. of DL-tryptophan were administered. The control subjects excreted an ether-extractable tryptophan-like compound after receiving DL-tryptophan, but excreted none of this compound after L-tryptophan.

Abnormal and varied excretion of tryptophan and nicotinic acid metabolites was found in three pellagrins after administration of 5 gm. of L-tryptophan per day. All three pellagrins excreted some of the ether-extractable tryptophan-like compound after receiving L-tryptophan. In two of these subjects, unusually high levels of "quinolinic acid" excretion were found following tryptophan administration.

The large amounts of "quinolinic acid" and of the ether-extractable tryptophan-like compound, which were found in the urine of one of the pellagrins before therapy, may have been due in part to azotemia, as evidenced by studies on two subjects with nephritis and uremia.

The administration of L-tryptophan to the patients with pellagra led to improvement in the clinical findings in two of the three subjects.

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BIBLIOGRAPHY

1. Sarett, H. P., and Goldsmith, G. A., *J. Biol. Chem.*, **167**, 293 (1947).
2. Perlzweig, W. A., Rosen, F., Levitas, N., and Robinson, J., *J. Biol. Chem.*, **167**, 511 (1947).
3. Sarett, H. P., and Goldsmith, G. A., *J. Biol. Chem.*, **177**, 461 (1949).
4. Sarett, H. P., *J. Biol. Chem.*, **182**, 671 (1950).
5. Henderson, L. M., *J. Biol. Chem.*, **178**, 1005 (1949).
6. Sarett, H. P., *J. Biol. Chem.*, **182**, 659 (1950).
7. Spies, J. R., and Chambers, D. C., *Anal. Chem.*, **20**, 30 (1948).
8. Porter, C. C., Clark, I., and Silber, R. H., *J. Biol. Chem.*, **167**, 573 (1947).
9. Greene, R. D., and Black, A., *Proc. Soc. Exp. Biol. and Med.*, **54**, 322 (1943).

THE EFFECT OF PYRIDOXINE UPON THE CONVERSION OF TRYPTOPHAN TO NICOTINIC ACID COMPOUNDS IN MAN*

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A deficiency of pyridoxine in the diets of rats, dogs, and mice has been shown to increase the excretion of xanthurenic acid after tryptophan administration (1-6) and to decrease the conversion of tryptophan to nicotinic acid compounds, as measured by urinary excretion of *N*¹-methylnicotinamide (*N*¹-Me) (6-8) and blood levels of pyridine nucleotides (9).

Studies in this laboratory on the metabolism of tryptophan in man have shown that the addition of pyridoxine to diets low in protein and B vitamins had no effect upon the conversion of large amounts of tryptophan (5 to 10 gm.) to nicotinic acid compounds (10, 11), and that supplementation of the diet with 2.5 or 5 gm. of L-tryptophan per day did not affect the level of excretion of 4-pyridoxic acid (12).

In the present studies pyridoxine compounds were added to the basal diets or to diets with small amounts of tryptophan added. The urines were analyzed for several nicotinic acid, tryptophan, and pyridoxine metabolites in an effort to elucidate further the rôle of pyridoxine in tryptophan metabolism in man.

EXPERIMENTAL

The subjects were ward patients similar to those employed in previous studies (10-13) and were maintained in a separate metabolism ward on the corn and wheat diets¹ which have been described (10, 13). Subjects 7 and 8 ate the same amounts of food each day, while Subject 11 occasionally failed to consume the entire diet. The recorded intakes are given in Tables I to III. The supplements were divided between dinner and supper and were not included in calculating the average intakes.

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¹ The unfortified corn products used in these studies were generously supplied by the Quaker Oats Company, through the courtesy of Dr. F. N. Peters, and the unenriched wheat products by General Mills, Inc., through the courtesy of Dr. F. C. Hildebrand and Dr. H. S. Faulkner.

24 hour urines were collected and pooled as previously described (10, 13). Analyses of creatinine, nitrogen, *N*¹-methylnicotinamide, 4-pyridoxic acid, and indoleacetic acid-like compounds were made by chemical methods, and nicotinic acid and tryptophan by microbiological methods (10, 13, 14). "Quinolinic acid" was estimated by the increase in nicotinic acid values obtained after acid autoclaving of the urine (10, 13, 15).

At the time of these experiments, little was known of the importance of the ether-extractable tryptophan-like metabolite (10-13) and this analysis is lacking in the study. The low values for tryptophan excretion in the three experiments suggest that little of the ether-extractable compound was present.

Many indole derivatives interfere with the method used for the measurement of indoleacetic-like compounds in urine, and the values obtained can only be considered as approximations, which are useful in showing relative changes (14, 16).

Results

Subject 7 (W. H., male, age 31, weight 64 kilos) was maintained on the corn diet for 22 days in the experiment shown in Table I. The addition of 3 mg. of pyridoxine per day was followed by a slight increase in excretion of *N*¹-Me and "quinolinic acid," and a small decrease in excretion of tryptophan. The *N*¹-Me excretion was highest in the first 2 days (Days 17 and 18) after removal of the pyridoxine supplement. The creatinine excretion on these days was higher than at any other time during the experiment. The addition of the pyridoxine supplement led to a significant decrease in excretion of indoleacetic acid-like compounds and a small increase in excretion of 4-pyridoxic acid (Table I).

Assuming that any effect of pyridoxine upon the conversion of tryptophan to nicotinic acid compounds might be seen more readily when a small amount of extra tryptophan was added to the diet, the experiment shown in Table II was carried out. Subject 8 (B. B., female, age 33, weight 67 kilos) was maintained on the corn diet to which 400 mg. of DL-tryptophan² were added each day. During the first 10 day period, the excretion of nicotinic acid compounds remained about the same, but the level of excretion of tryptophan, indoleacetic acid compounds, and 4-pyridoxic acid gradually increased (Table II).

Subject 8 then received 3 mg. of pyridoxine hydrochloride per day for an 8 day period. The results in Table II show that the addition of the pyridoxine had no effect upon the excretion of nicotinic acid, "quinolinic acid," *N*¹-Me, or tryptophan. A slight increase in excretion of 4-pyridoxic

² The DL-tryptophan was generously provided by the Winthrop-Stearns Chemical Company, Inc., through the courtesy of Mr. Kenneth Smoot.

TABLE I

Effect of Administration of Pyridoxine upon Daily Excretion of Nicotinic Acid and Tryptophan Compounds by Subject 7

Diet	Day No.	Creatinine	Nitrogen	Nicotinic acid		Ni-Me	Tryptophan	Indole-acetic-like compounds	4-Pyridoxic acid
				Before acid auto-claving	After acid auto-claving				
		gm.	gm.	mg.	mg.	mg.	mg.	mg.	mg.
Corn (2500 calories, 37 gm. protein, 6 mg. nicotinic acid)	1-2	1.12	4.3	1.3	2.1	2.8	16	8	2.1
	3-4	1.05	4.0	1.2	1.9	1.7	17	7	1.7
	5-6	0.98	3.8	1.2	1.9	1.9	20	6	1.9
	7-8	1.13	4.3	0.9	1.9	2.0	21	6	2.1
Corn + 3 mg. pyridoxine HCl per day	9-10	0.91	3.6	1.1	2.0	1.7	16	6	2.8
	11-12	0.93	3.5	1.0	2.0	1.8	13	1	1.8
	13-14	1.10	4.2	1.2	2.4	2.4	15	4	2.6
	15-16	0.93	3.6	1.2	2.2	2.0	13	1	2.4
Corn	17-18	1.26	4.0	1.3	2.4	3.2	16	1	2.0
	19-20	1.10	3.8	1.0	1.9	2.0	12	2	1.5
	21-22	0.91	3.3	0.8	1.4	1.5	13	6	1.4

TABLE II

Effect of Administration of Pyridoxine and Tryptophan upon Daily Excretion of Related Compounds by Subject 8

Diet	Day No.	Creatinine	Nitrogen	Nicotinic acid		Ni-Me	Tryptophan	Indole-acetic-like compounds	4-Pyridoxic acid
				Before acid auto-claving	After acid auto-claving				
		gm.	gm.	mg.	mg.	mg.	mg.	mg.	mg.
Corn* + 400 mg. DL-tryptophan per day	1-2		3.3	1.1	2.3	2.4	16	3	0.7
	3	1.17	3.6	0.9	1.8	2.5	18	7	1.6
	5-6								
	7-8	1.19	4.1	1.1	2.2	2.3	19	7	1.4
	9-10	1.23	3.6	0.7	1.7	1.8	20	12	2.0
Corn + 400 mg. DL-tryptophan + 3 mg. pyridoxine HCl per day	11-12	1.23	3.6	0.8	2.2	2.1	20	8	2.4
	13-14	1.19	3.3	1.0	2.4	1.8	18	4	1.7
	15-16	1.23	3.4	0.9	2.3	1.8	18	2	2.1
	17-18	1.14	3.4	0.7	2.4	1.7	17	4	2.2
Corn + 400 mg. DL-tryptophan per day	19-20	1.18	4.2	0.9	1.9	1.9	20	13	2.2
	21-22	1.19	3.7	1.0	2.2	2.1	20	10	1.8
	23-24	1.16	3.6	0.9	2.9	2.0	20	5	1.1
Corn	25-26	1.16	3.5	1.0	2.1	1.7	11	1	0.9
	27-28	1.19	4.1	1.1	1.8	2.0	12	5	2.0
	29-30	1.21	4.1	1.1	2.1	1.8	13	1	1.1

* Average intake, 2500 calories, 37 gm. of protein, 6 mg. of nicotinic acid.

acid and a significant decrease in excretion of indoleacetic acid-like compounds were noted. When the added pyridoxine was removed from this diet (Days 19 to 24), the excretion of nicotinic acid compounds and of tryptophan was not changed but the indoleacetic acid excretion was increased. The removal of the tryptophan supplement from the diet (Days 25 to 30) led to no change in the excretion of nicotinic acid compounds and to a decrease in excretion of tryptophan and of indoleacetic acid compounds (Table II).

TABLE III

Effect of Administration of Gelatin, Tryptophan, and Pyridoxine upon Daily Excretion of Related Compounds by Subject 11

Diet	Day No.	Creatinine	Nitrogen	Nicotinic acid		N ^L -Me	Tryptophan	4-Pyridoxic acid
				Before acid auto-claving	After acid auto-claving			
		gm.	gm.	mg.	mg.	mg.	mg.	mg.
Wheat (2730 calories, 44 gm. protein, 6.2 mg. nicotinic acid)	1-2	0.73	3.5	0.6	1.7	2.0	11	0.9
	3-4	0.74	3.3	0.5	1.3	1.9	12	1.3
	5-6	0.74	3.8	0.7	1.7	1.4	11	1.1
	7-8	0.75	3.7	0.5	1.7	1.4	12	1.2
Wheat + 20 gm. gelatin + 200 mg. DL-tryptophan per day	9-10	0.80	4.7	0.6	1.7	1.9	15	1.5
	11-12	0.63	4.8	0.4	1.3	1.9	12	1.1
	13-14	0.72	5.9	0.6	1.7	2.1	13	1.7
	15-16	0.71	6.4	0.5	1.5	2.5	15	1.3
	17-18	0.60	5.7	0.7	1.5	1.9	11	1.1
	19-20	0.71	6.2	0.7	1.4	1.8	11	1.0
Wheat + 20 gm. gelatin + 200 mg. DL-tryptophan + 5 mg. pyridoxine HCl per day	21-22	0.71	5.9	0.3	1.0	1.4	12	3.3
	23-24	0.71	5.2	0.3	1.0	1.4	12	3.4
	25-26	0.74	5.1	0.4	1.1	1.4	14	3.5
	27-28	0.79	6.0	0.4	1.1	1.3	15	3.8
Wheat + 20 gm. gelatin + 200 mg. DL-tryptophan per day	29-30	0.79	7.0	0.4	1.1	1.3	16	2.0
	31-32	0.92	7.0	0.9	1.9	1.4	16	1.5
	33-34	0.88	6.6	0.6	1.5	1.2	15	1.2
Wheat + 200 mg. DL-tryptophan per day	35-36	0.94	5.9	0.7	1.8	1.8	17	1.3
	37-38	0.95	4.4	0.6	1.7	2.0	16	0.7

The experiments given in Tables I and II, as well as those reported previously (10), show that added pyridoxine has little effect, if any, upon the excretion of nicotinic acid compounds and of tryptophan on control diets or when small or large amounts of tryptophan are added to the diet.

Since pyridoxine is needed for protein metabolism (4, 17), it was considered that extra protein in the diet would increase the need for pyridoxine and might show the rôle of pyridoxine in the conversion of tryptophan to nicotinic acid. Subject 11 (A. D., female, age 32, weight 58 kilos) received

the wheat diet, which was later supplemented with 20 gm. of gelatin³ and 200 mg. of DL-tryptophan per day. The gelatin and the small amount of tryptophan had virtually no effect upon the excretion of nicotinic acid, "quinolinic acid," tryptophan, and 4-pyridoxic acid, but led to a small increase in excretion of *N*¹-Me. The 3 gm. of nitrogen in the gelatin supplement led to an average increase in nitrogen excretion of 2 gm. per day. Other subjects who had received 20 gm. of gelatin supplements retained more of the extra nitrogen than did Subject 11 (13).

When pyridoxine was added with the above supplements (Days 21 to 28, Table III), a decreased excretion of nicotinic acid, "quinolinic acid," and *N*¹-Me was noted, which could not be correlated with any change in tryptophan or nitrogen balance. This has sometimes been found in other subjects who were receiving large amounts of DL-tryptophan when the pyridoxine was added (10). The pyridoxine addition increased the excretion of 4-pyridoxic acid (Table III).

The removal of the pyridoxine (Days 29 to 34, Table III) led to a significant change in nitrogen balance. The increased loss of urinary nitrogen was accompanied by increased excretion of nicotinic acid and "quinolinic acid," and no change in excretion of *N*¹-Me or tryptophan.

The removal of the gelatin for the final period in the experiment resulted in an increase in excretion of *N*¹-Me and a decrease in output of urinary nitrogen. This change in *N*¹-Me excretion has not been found in other subjects after removal of gelatin or glycine supplements from the diet (13). The gradual lowering of the 4-pyridoxic acid values after Day 28 was due primarily to removal of the pyridoxine supplement rather than to the subsequent changes in the diet.

DISCUSSION

Pyridoxine deficiency has been shown to decrease the ability of rats and mice to convert tryptophan to nicotinic acid, as measured by the excretion of nicotinic acid and *N*¹-Me after administration of tryptophan (6-9). Severe caloric restriction of a complete diet also impaired the ability of the rat to convert tryptophan to *N*¹-Me, but not to the same extent as has been found in pyridoxine deficiency (18). Spector (19) has shown that the omission of pyridoxine from the diet had no significant effect upon the conversion of tryptophan to nicotinic acid and *N*¹-Me in forced feeding in rats. It appears, therefore, that findings in pyridoxine deficiency are due, in part, to the small food intake and poor growth of the deficient animals.

Experiments on the conversion of tryptophan to nicotinic acid compounds by man have been conducted with basal diets low in tryptophan,

³ The gelatin was generously supplied by the Knox Gelatine Protein Products, Inc., through the courtesy of Dr. D. Tourtellotte.

protein, and B vitamins (10-13). The addition of extra pyridoxine or pyridoxal to these diets, which were maintained at the same caloric level throughout each experiment, led either to no change or to a decrease in conversion of 5 or 10 gm. supplements of DL-tryptophan to nicotinic acid compounds (10). In the present experiments, the addition of pyridoxine to the corn diet had little effect upon excretion of nicotinic acid compounds, and no effect upon the conversion of a small tryptophan supplement to nicotinic acid, as measured by excretion of nicotinic acid compounds. However, the pyridoxine supplement did influence tryptophan metabolism as evidenced by the decrease in excretion of indoleacetic acid-like compounds. Recent experiments⁴ have shown that the tryptophan metabolite which can be extracted from the acidified urine with ether, and can replace L-tryptophan for growth of *Lactobacillus arabinosus* (10-13), gives the same ferric chloride color reaction as does indoleacetic acid. This unidentified metabolite may be responsible for the urorosein reaction (20).

The addition of 2.5 or 5 gm. of L-tryptophan or of 20 gm. of gelatin or glycine to the diet had no effect upon the excretion of 4-pyridoxic acid by man (12, 13). However, the addition of both gelatin and L-tryptophan to the diet has been found to decrease the excretion of 4-pyridoxic acid⁵ (13). These results suggest that, although a complete protein increases the need for pyridoxine (4, 17), the addition of an incomplete protein or of a single amino acid may have little effect upon pyridoxine metabolism in man.

The data on the interrelationship of tryptophan, nicotinic acid, and pyridoxine metabolism in man are incomplete (10-13) and lack particularly information on the effects of pyridoxine deficiency in man. However, if pyridoxine is directly involved in the conversion of tryptophan to nicotinic acid, it is surprising that the addition of pyridoxine to a diet low in B vitamins has no effect upon the amount of conversion of tryptophan to nicotinic acid, and that large tryptophan supplements have no effect upon excretion of 4-pyridoxic acid.

The present information indicates that pyridoxine is involved in at least one phase of tryptophan metabolism in man (effect on the excretion of indoleacetic acid-like compounds) but gives no definite evidence of a direct rôle in the conversion of tryptophan to nicotinic acid.

SUMMARY

The addition of pyridoxine to a corn diet low in protein, tryptophan, and B vitamins had little effect upon the excretion of nicotinic acid, "quino-

⁴ Sarett, H. P., and Loeb, J. M., unpublished data.

⁵ This could not be shown when gelatin and DL-tryptophan were added to the diet of Subject 11 (Table III), since the metabolism of DL-tryptophan leads to the excretion of compounds which interfere with the assay of 4-pyridoxic acid (10, 12).

linic acid," or *N*¹-methylnicotinamide by man, and no effect upon the conversion of a small amount of added tryptophan to these nicotinic acid compounds, as measured by the urinary excretion of the above compounds.

The pyridoxine supplement led to a significant decrease in excretion of indoleacetic acid-like compounds by subjects on the basal corn diet or on the diet supplemented with tryptophan.

The excretion of nicotinic acid, "quinolinic acid," and *N*¹-methylnicotinamide by a subject receiving a basal diet supplemented with gelatin and tryptophan was decreased when pyridoxine was also added to the diet.

The rôle of pyridoxine in the metabolism of tryptophan in man is discussed.

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BIBLIOGRAPHY

1. Lepkovsky, S., Roboz, E., and Haagen-Smit, A. J., *J. Biol. Chem.*, **149**, 195 (1943).
2. Axelrod, H. E., Morgan, A. F., and Lepkovsky, S., *J. Biol. Chem.*, **160**, 155 (1945).
3. Reid, D. F., Lepkovsky, S., Bonner, D., and Tatum, E. L., *J. Biol. Chem.*, **155**, 299 (1944).
4. Miller, E. C., and Baumann, C. A., *J. Biol. Chem.*, **157**, 551 (1945).
5. Porter, C. C., Clark, I., and Silber, R. H., *J. Biol. Chem.*, **167**, 573 (1947).
6. Rosen, F., Huff, J. W., and Perlzweig, W. A., *J. Nutr.*, **33**, 561 (1947).
7. Schweigert, B. S., and Pearson, P. B., *J. Biol. Chem.*, **168**, 555 (1947).
8. Bell, G. H., Scheer, B. R., and Deuel, H. J., Jr., *J. Nutr.*, **35**, 239 (1948).
9. Ling, C.-T., Hegsted, D. M., and Stare, F. J., *J. Biol. Chem.*, **174**, 803 (1949).
10. Sarett, H. P., and Goldsmith, G. A., *J. Biol. Chem.*, **177**, 461 (1949).
11. Sarett, H. P., *J. Biol. Chem.*, **182**, 671 (1950).
12. Sarett, H. P., and Goldsmith, G. A., *J. Biol. Chem.*, **182**, 679 (1950).
13. Sarett, H. P., *J. Biol. Chem.*, **182**, 659 (1950).
14. Tang, Y. W., and Bonner, J., *Arch. Biochem.*, **13**, 11 (1947).
15. Henderson, L. M., *J. Biol. Chem.*, **178**, 1005 (1949).
16. Sarett, H. P., *Proc. Soc. Exp. Biol. and Med.*, **69**, 494 (1948).
17. Conger, T. W., and Elvehjem, C. A., *J. Biol. Chem.*, **138**, 555 (1941).
18. Junqueira, P. B., and Schweigert, B. S., *J. Biol. Chem.*, **175**, 535 (1948).
19. Spector, H., *J. Biol. Chem.*, **173**, 659 (1948).
20. Herter, C. A., *J. Biol. Chem.*, **4**, 253 (1908).

THE ABSENCE OF α -AMINOADIPIC ACID IN CHOLERA VIBRIO

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Blass and Macheboeuf reported that they had isolated α -aminoadipic acid from cholera *Vibrio* (1). On repeating the experiment later, they tested the purity of the isolated product by means of paper chromatograms developed with phenol and retracted their earlier claim (2). However, Borsook *et al.* found that α -aminoadipic acid and glutamic acid gave the same spot on two-dimensional paper chromatograms developed with phenol and *s*-collidine (3). The retraction of Blass and Macheboeuf was therefore based on a method which does not differentiate between the two dicarboxylic amino acids.

By using the starch chromatographic method of Stein and Moore (4), Borsook *et al.* were able to separate α -aminoadipic acid from other amino acids, including glutamic acid (3). In this present study an attempt is made by the same technique to determine whether α -aminoadipic acid is present in the cholera *Vibrio*.

Method

Starch columns (0.9 cm. \times 30 cm.) were prepared according to the Stein and Moore method. C^{14} -labeled α -aminoadipic acid¹ in a mixture of amino acids was put on a column and eluted with 1 part *n*-butanol, 2 parts *n*-propanol, and 1 part 0.1 *N* hydrochloric acid (1:2:1 solvent). The position of the radioactivity together with the intensity of the ninhydrin color in the eluate gave a reproducible peak at between 40 and 42 ml. for the α -aminoadipic acid. This is in good agreement with the results of Moore and Stein (5).

Later it was found that proline can be used as a marker, since it is eluted immediately after α -aminoadipic acid (peak at about 50 ml. of eluate) and can be detected by its yellow color with ninhydrin.

Washed and killed cholera organisms which had been raised on potato starch medium² were hydrolyzed by refluxing with 20 parts of 20 per cent

¹ The radioactive α -aminoadipic acid was obtained through the courtesy of Dr. Peter H. Lowy. The C^{14} used in this investigation was supplied by the Monsanto Chemical Company, Clinton Laboratories, and obtained on allocation from the United States Atomic Energy Commission.

² Supplied through the generosity of Dr. H. A. Dettwiler of Eli Lilly and Company, Indianapolis.

hydrochloric acid for 24 hours on an oil bath. The hydrolysate was filtered and evaporated nearly to dryness *in vacuo*. A quantity equivalent to 2.4 mg., dry weight, dissolved in 0.4 ml. of 1:2:1 solvent was put on a starch column, and the amino acids eluted with the same solvent on an automatic fraction-collector. The 0.5 ml. fractions were tested by the Moore and Stein photometric ninhydrin method (6). No evidence of the presence of α -aminoadipic acid was found.

In other experiments the dicarboxylic amino acids were separated from the cholera hydrolysate by precipitation with calcium hydroxide and absolute ethyl alcohol. Calcium was removed with oxalate and the amino acids chromatographed on a starch column. Again no α -aminoadipic acid was found.

Since there existed the possibility that the cholera *Vibrio* formed α -aminoadipic acid and excreted it into the culture medium, a sample of potato starch medium, after the harvesting of the cholera organisms for cholera vaccine,² was chromatographed on a starch column. As in the other experiments, there was no α -aminoadipic acid.

SUMMARY

Cholera *Vibrio* cultured on potato starch medium does not accumulate α -aminoadipic acid.

BIBLIOGRAPHY

1. Blass, J., and Macheboeuf, M., *Helv. chim. acta*, **29**, 1315 (1946).
2. Blass, J., and Macheboeuf, M., *Bull. Soc. chim. biol.*, **29**, 903 (1947).
3. Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H., *J. Biol. Chem.*, **176**, 1383 (1948).
4. Stein, W. H., and Moore, S., *J. Biol. Chem.*, **176**, 337 (1948).
5. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **178**, 53 (1949).
6. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **176**, 367 (1948).

THE APPLICATION OF A TREATMENT OF CONSECUTIVE REACTIONS TO THE ACIDIC HYDROLYSIS OF RIBONUCLEIC ACIDS

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An important pathway for investigations concerning the structures of ribonucleic acids was opened by Jones and coworkers (1) with their discovery that the phosphate of nucleic acid from yeast hydrolyzed at two distinct rates. This discovery has been employed by later workers (2-5) to determine the partition of purine and pyrimidine nucleotides in ribonucleic acids. However, variations in the conditions of hydrolysis and in methodology have given values of the rate constants that are difficult to correlate. Furthermore, the partition between purines and pyrimidines in nucleic acid as obtained by acidic hydrolysis has been difficult to correlate with the partition obtained by the newer procedures of microbiological assay (6-8) and of paper chromatography (9). Therefore, in order that the data which are obtained concerning the structure of nucleic acids by the use of acidic hydrolysis might be brought to greater usefulness and meaning, an intensive study of the kinetics of hydrolysis has been undertaken. In particular, two objectives were sought: (1) to determine whether significant differences exist between the kinetics of labile phosphate of nucleic acid and of phosphate of purine nucleotides, as well as between the kinetics of stable phosphate of nucleic acid and of phosphate of pyrimidine nucleotides, and (2) to standardize a simple procedure for the accurate partition of the labile from the stable phosphate of nucleic acid, in the expectation that such a partition will be a useful addition to an analytical scheme for the characterization of ribose nucleic acids.

EXPERIMENTAL

A commercial preparation of nucleic acid from yeast (Schwarz) was purified by accepted procedures. The nucleic acid was dissolved by the addition of a dilute solution of sodium hydroxide and the pH adjusted to 7.0. The solution was dialyzed. The nucleic acid which remained in the dialysis bags was recovered by lyophilization. In those experiments designed to study the fragments which result from ribonuclease action, ribonuclease was added to a solution of the purified dialyzed nucleic acid at pH 7.0. Dialysis was continued and the dialyzable and residue fractions were isolated by lyophilization.

Nucleotides were isolated according to the procedures described by Levene and coworkers (10).

Triplicate analyses for phosphate were performed by the use of the method of Fiske and Subbarow (11). Statistical analysis of the data indicated that 95 per cent of the determinations were within 1.7 per cent of the true value.

Owing to the precipitation of nucleic acids in acidic molybdate solutions, the analysis for inorganic phosphate in the presence of nucleic acids is difficult. In such cases inorganic phosphate is coprecipitated. The extent of coprecipitation of inorganic phosphate may be estimated by the addition of known amounts of inorganic phosphate. When the inorganic phosphate is less than 3 per cent of the total phosphate, coprecipitation may be as high as 40 per cent. When the inorganic phosphate is 30 per cent or more of the total phosphate, the relative extent of coprecipitation is negligible. Since filtration removes considerable color from visually clear solutions of molybdenum blue, turbidity due to precipitated nucleic acids is removed by centrifugation.

Hydrolyses were conducted in 0.500 N solution of hydrochloric acid with 0.1 to 0.13 mg. of P per ml. and in sealed tubes. At zero time the sealed tubes were immersed into a vigorously boiling water bath. At known intervals, four or five tubes were removed from the bath during the 1st hour of a hydrolysis. Four more were removed at 105, 150, 240, and 285 minutes. After removal from the bath, a tube was placed in ice water. The tube, when cold, was wiped dry, shaken, opened, and sampled immediately. The hydrolysis curve for a sample of ribonucleic acid from yeast is illustrated in Fig. 1.

The first step in the calculation of the rate constants is the computation of a linear regression line through the last four points of the curve (Fig. 1). Thus, if Y_i is the inorganic phosphate in mg. of P per tube at time t_i , then for the last four points $Y(t) = a + bt$. The intercept a determines the partition between labile and stable phosphate. The fraction of the total P which is labile is equal to the expression $(a - Y_0)/c_0$ where Y_0 is the inorganic phosphate initially present and c_0 is the phosphate per tube.

For the calculation of the first order rate constant, assume that the stable phosphate of nucleic acid is hydrolyzed according to the first order rate law (12), and assume further that the center of the regression line, at 3.25 hours, is tangent to the true rate curve. Let $K_s = b/(c_0 - a)$; $K'_s =$ the true first order rate constant, expressed with natural logarithms; $\alpha =$ the intercept of the true rate curve. Then it can be shown that $K'_s = K_s/(1 - 3.25 K_s)$. It can also be shown that the ratio $(c_0 - \alpha)/(c_0 - a)$ equals 1.007; so that the use of a , instead of α , to determine the partition between labile and stable phosphate introduces a negligible error.

After having established that the same two rates of hydrolysis of phosphate are found in the nucleic acids of yeast and of pancreas, in certain fragments which result from ribonuclease action, and in the nucleotides, it is possible to simplify the determinations of the partition of phosphate. A single tube is hydrolyzed for 1.75 hours. Samples are taken from the tube for the analysis of inorganic and of total phosphate. The use of an average value of K_s permits the calculation of a , since $a + 1.75K_s (c_0 - a) = Y_{1.75}$. The value of $1.75K_s$, determined from several substances, is 0.0613.

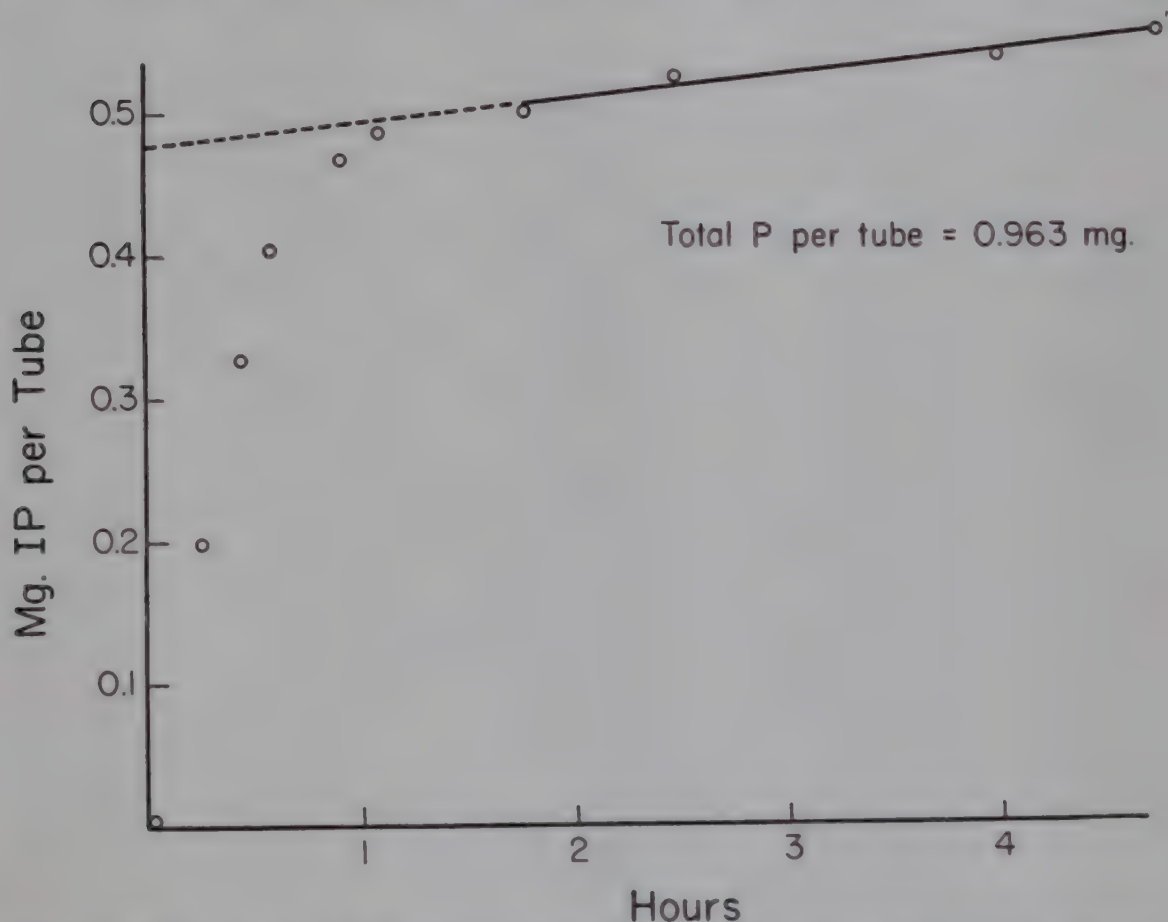


FIG. 1. Hydrolysis of ribonucleic acid from yeast. IP = inorganic phosphate expressed as phosphorus.

As a demonstration of precision, six such single tube hydrolyses were run on one preparation. The values of labile phosphate that were obtained were 51.3, 52.0, 51.2, 51.7, 51.0, and 50.3 per cent. Both $Y_{1.75}$ and c_0 were determined in each tube; thus the analyses were independent, except for the common value of K_s .

The second regression line is fitted to the data for labile phosphate obtained during the 1st hour of the hydrolysis. $Y^*_i = Y_i - bt_i - Y_0$ = the amount of labile phosphate hydrolyzed at time t_i . The function $f(Y^*_i) = \ln((a - Y_0)/(a - Y^*_i))$ is computed for each point, and the regression of $f(Y^*)$ on t is determined, $f(Y^*) = c + dt$. The zero time point is not in-

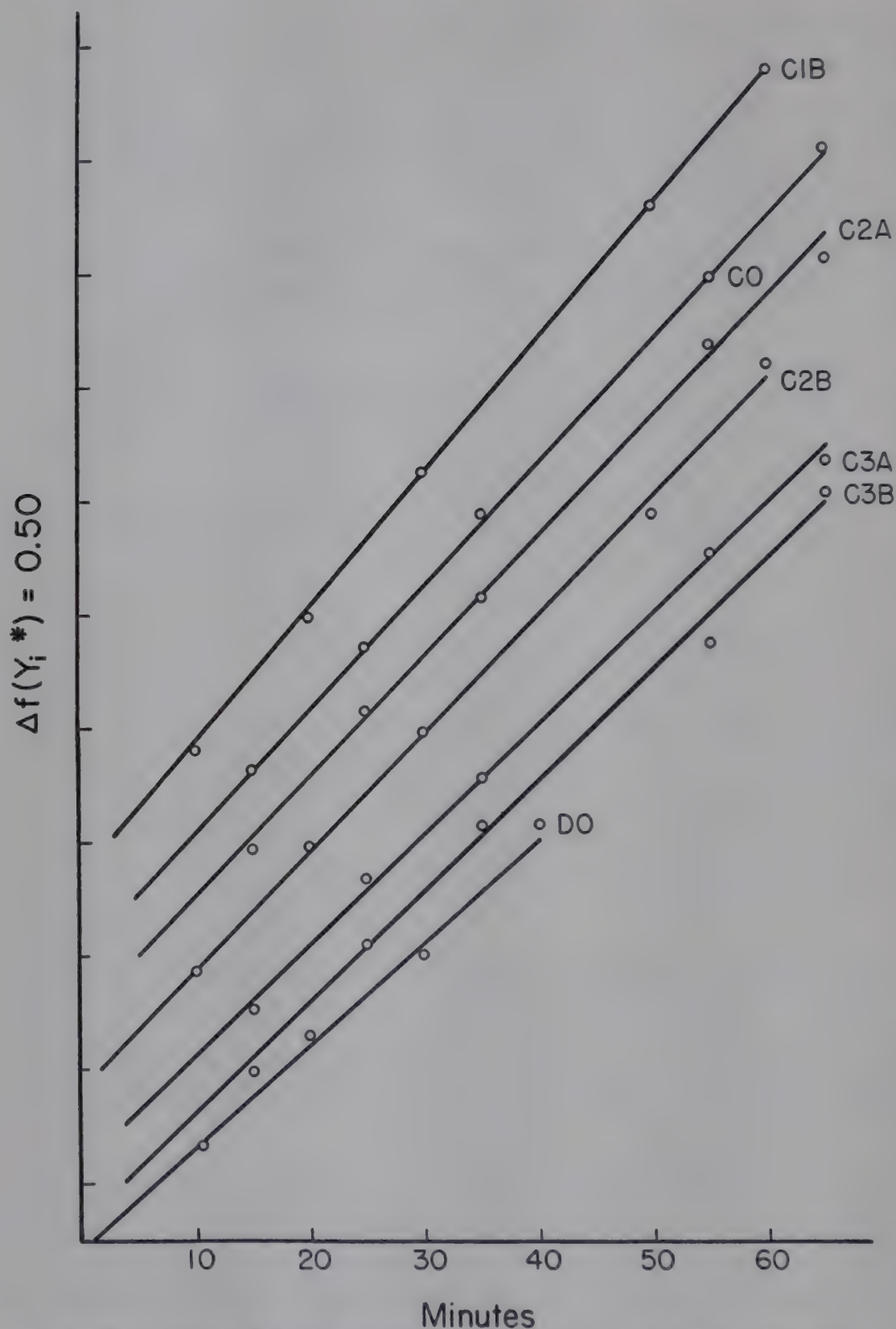


FIG. 2. Hydrolysis of labile phosphate of two types of ribonucleic acid and of their dialyzable and residue fractions before and after the action of ribonuclease. DO is ribonucleic acid from pancreas. CO is ribonucleic acid from yeast. C1B and C2B are successive fractions obtained by dialysis at 48 hour intervals from CO. C3B is the residue after removal of fractions C1B and C2B from CO. C2A is a dialyzable fraction obtained by the action of ribonuclease on dialyzed CO. C3A is

cluded in the regression line because of temperature lag inside of the tubes on immersion in the water bath. This factor contributes to an observed induction period, which results in the intercept, $-c/d$, being approximately 5 minutes in each of the many cases studied. Hereafter $-c/d$ will be designated as t^* . The induction period will be discussed in detail in a later section.

The first order rate constant of the labile phosphate expressed with natural logarithms is given directly by d . An average value for the half life is 13 minutes; consequently, $(1/2)^{105/13}$, or 0.004, is the fraction of labile

TABLE I

*First Order Rate Constants Expressed with Natural Logarithms, and Values of t^**

	d	t^*	K_1
	hrs. ⁻¹	min.	hrs. ⁻¹
Ribonucleic acid from yeast.....	3.3	4.8	0.047
Test dialysate.....	3.1	5.2	0.033
“ residue.....	2.9	3.5	0.050
First control dialysate.....	3.6	3.3†	0.042
Second “ “.....	3.1	1.6†	0.032
Control residue.....	2.9	4.0	0.033
Ribonucleic acid from pancreas (Preparation 1).....	2.7	0.9†	0.050
“ “ “ “ (“ 2).....	3.0	5.5	0.057
Adenosine-3-phosphate.....	2.9	4.8	
Equimolar mixture of adenylic and cytidylic acids....	3.4	5.5	0.041
“ “ “ “ “ “ “ “.....	3.0	3.5	0.041
“ “ “ “ “ “ “ “.....	3.1	5.5	0.038
Cytidylic acid.....			0.0394
Ammonium uridylate.....			0.0322

† Obtained by a special procedure; see the text.

phosphate remaining when the first point of the first regression line is measured.

Table I contains the values of t^* and of the rate constants. Figs. 2, 3, and 4 demonstrate that the first order functions of the various hydrolyses are linear with time. Fig. 5 shows that, regardless of contamination, guanylic acid is hydrolyzed to analytical completion in 90 minutes at approximately the same rate at which adenylic acid is hydrolyzed. In other concentrations of acid, the rates for guanylic and adenylic acids are

the residue which remained after removal of C2A from CO. The points are experimental. The lines are the regression lines, $f(Y^*) = c + dt$. In order to avoid overlapping, the scale of ordinate has no origin. 1 scale unit indicates a change of 0.50 in $f(Y^*)$. The lower terminus of each line has as abscissa the value t^* , which is equal to $-c/d$. The figure shows that $f(Y^*)$ is linear with time over the last two-thirds of the reaction.

the same (13-15), although Kobayashi (3) prepared samples of the two acids which gave considerably different rates from each other.

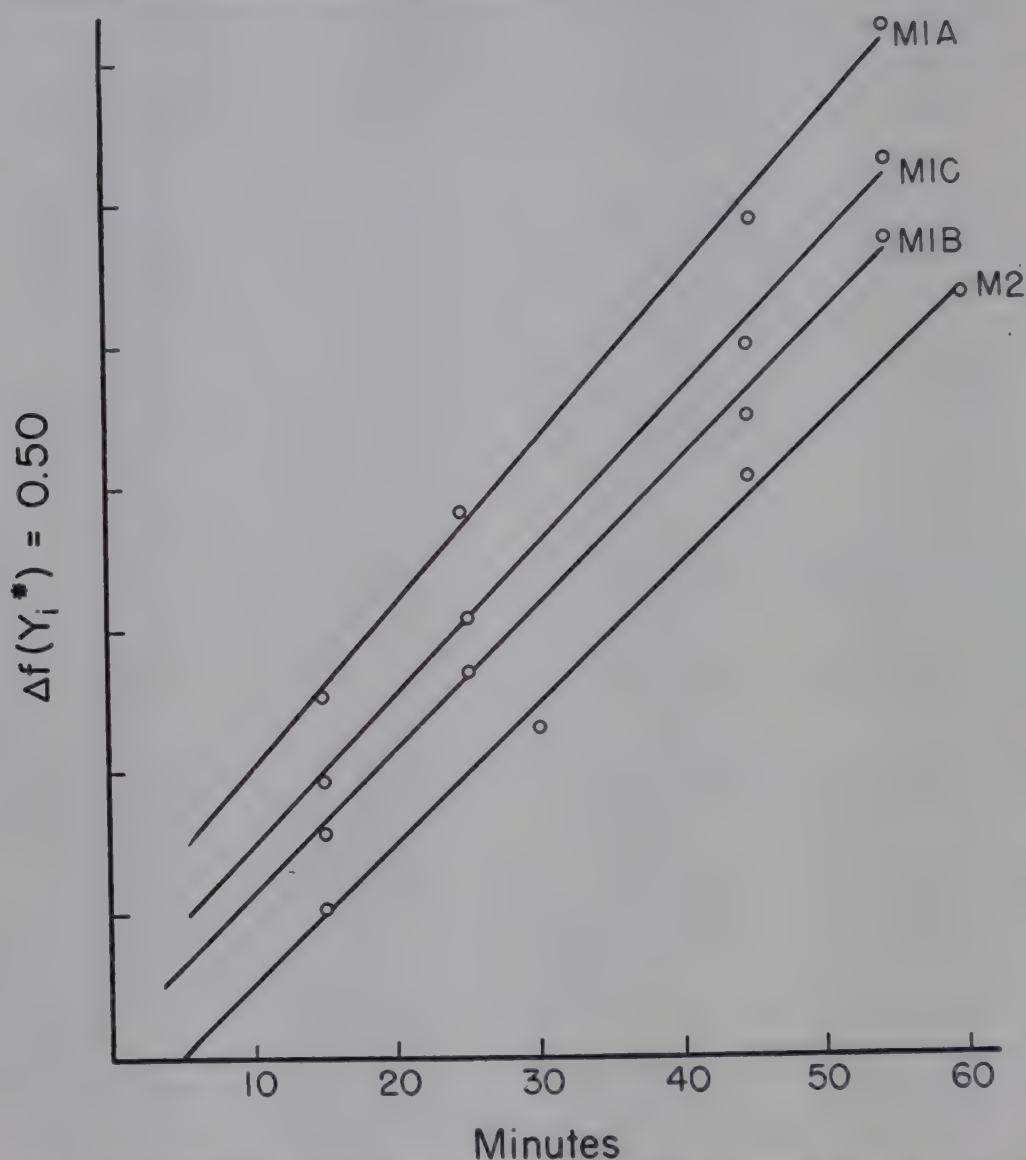
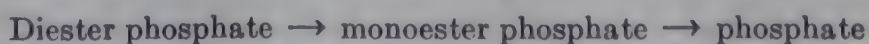


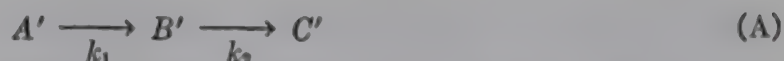
FIG. 3. Hydrolysis of adenylic acid and of mixtures of adenylic and cytidylic acids. M2 is 99 per cent pure adenylic acid. M1A, M1B, and M1C are equimolar mixtures of adenylic and cytidylic acids. M1A was moistened with water, then dried *in vacuo* for 27 hours at 111° over phosphorus pentoxide. M1B was neutralized with sodium hydroxide, evaporated to dryness at room temperatures, and finally dried *in vacuo* for 20 hours at 111° over phosphorus pentoxide. During the preliminary evaporation of sample M1B, 9.8 per cent of the total phosphate was hydrolyzed; however, the presence of this large amount of inorganic phosphate did not affect the estimate of the rate constant for labile phosphate.

DISCUSSION

Numerous recorded titrations of nucleic acids from yeast indicate the presence of diester phosphate. The hydrolysis of both labile and stable phosphate from nucleic acid may be thought to follow the path:



This discussion will be particularly concerned with labile phosphate. Consider the reaction



Let A_0 be the initial concentration of substance A' , and A be the concentration at time t . Similarly B and C will represent the concentrations

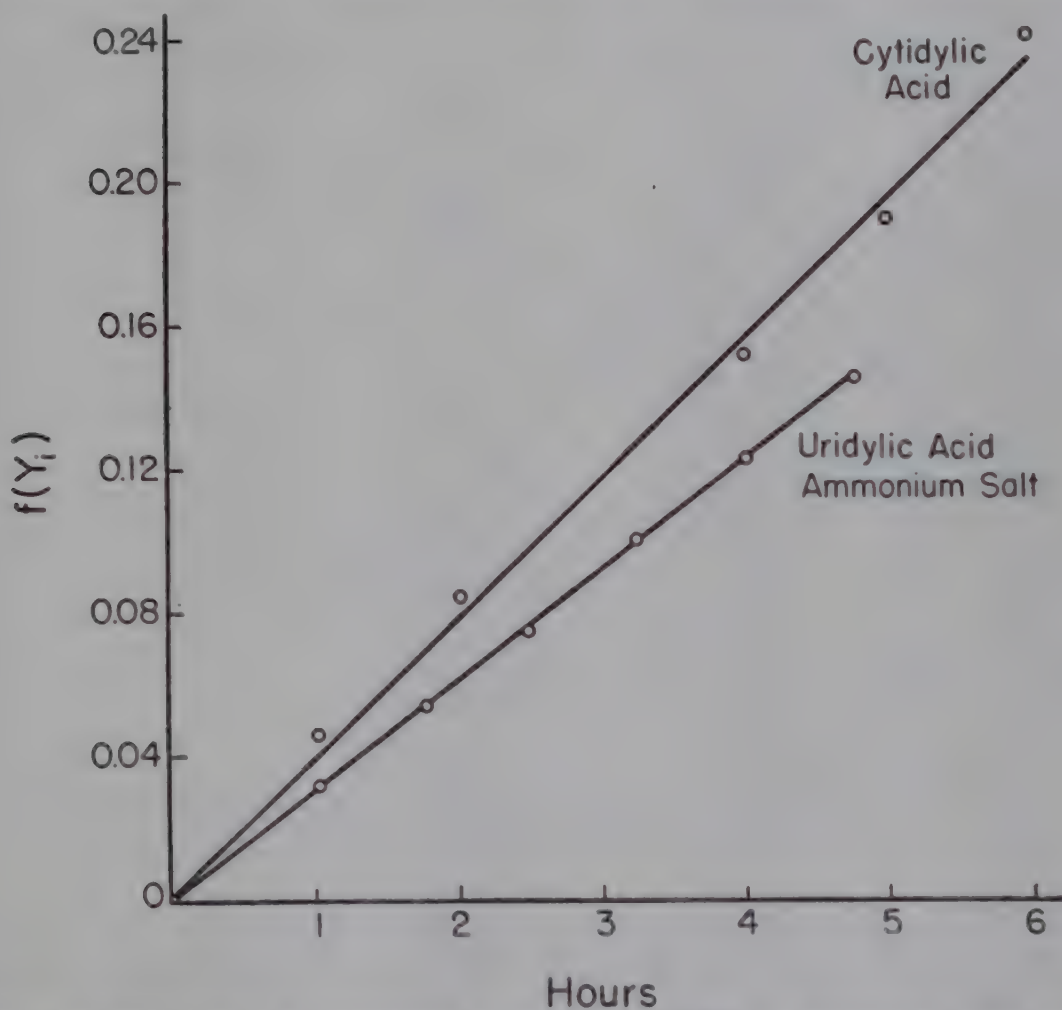


FIG. 4. Hydrolysis of pyrimidine nucleotides. $f(Y_i)$ is described in the text

of substances B' and C' . Integration of the rate equations gives

(a) $k_1 \neq k_2$

$$B = \frac{k_1 A_0}{k_2 - k_1} (e^{-k_1 t} - e^{-k_2 t}) \quad (1)$$

$$C = A_0 \left[1 - \frac{k_2}{k_2 - k_1} e^{-k_1 t} + \frac{k_1}{k_2 - k_1} e^{-k_2 t} \right] \quad (2)$$

(b) $k_1 = k_2$

$$B = k_1 A_0 t e^{-k_1 t} \quad (3)$$

$$C = A_0 [1 - e^{-k_1 t} (1 + k_1 t)] \quad (4)$$

It may be noted that equation (2) is symmetrical with respect to the values of k .

Since it is demonstrated that $f(Y^*)$ is linear with time (Fig. 2), it is of interest to examine the circumstances under which $\ln [(A_0)/(A_0 - C)]$ would

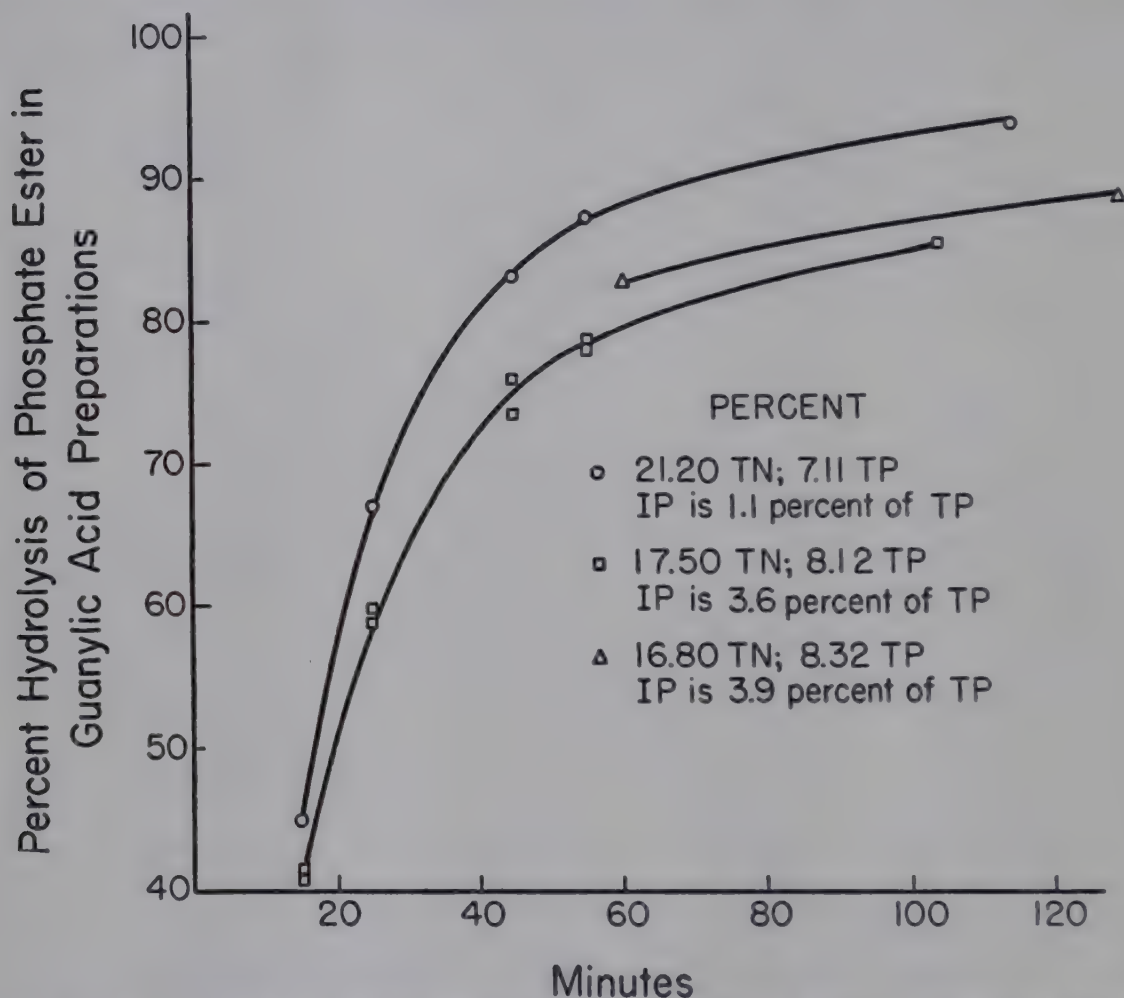


FIG. 5. Hydrolysis of three different preparations of guanylic acids of varying degrees of purity. TN = total nitrogen. IP is inorganic phosphate expressed as phosphorus. TP is total phosphorus.

be linear with time, given mechanism (A). This is conveniently done by forming the function $\ln [(A_0)/(A_0 - C)]$, with equation (2) as the explicit form for C .

$$\ln \left[\frac{A_0}{A_0 - C} \right] = -\ln \left[\frac{k_2}{k_2 - k_1} e^{-k_1 t} - \frac{k_1}{k_2 - k_1} e^{-k_2 t} \right] \quad (5)$$

The right side is still symmetrical with respect to k . If $k_1 t \gg k_2 t$, the right side reduces to

$$\ln \left[\frac{A_0}{A_0 - C} \right] = k_2 t - \ln \left[\frac{k_1}{k_1 - k_2} \right] \quad (6)$$

For k_2 larger than k_1 , the same expression, with the subscripts of k inverted,

is obtained. Let R be the ratio of the larger of the two values of k to the smaller. Then increasing R , or increasing t , makes equation (6) an increasingly better approximation to equation (5). Actually equation (6) is a good approximation to equation (5) for even low values of R if t is greater

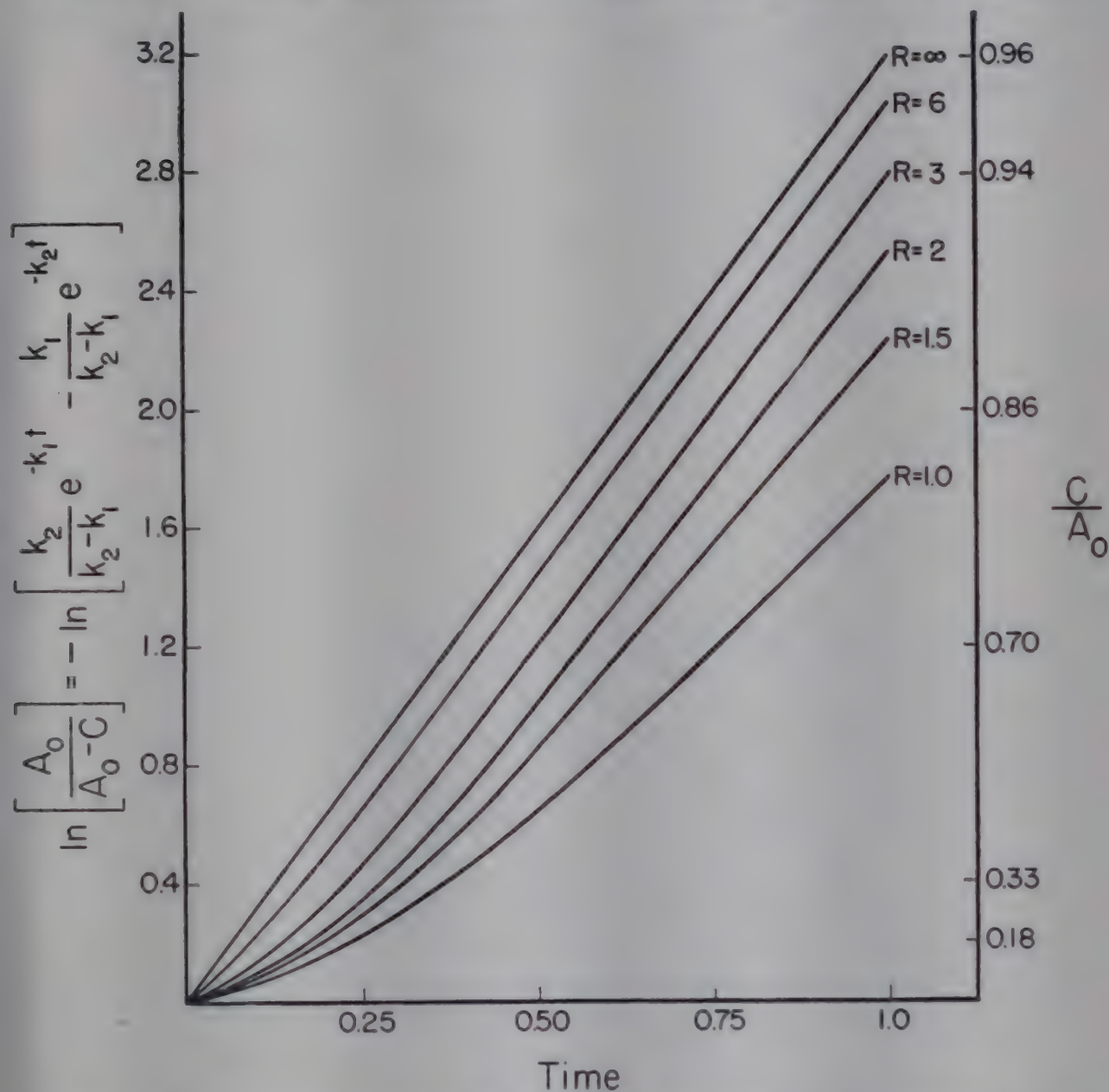
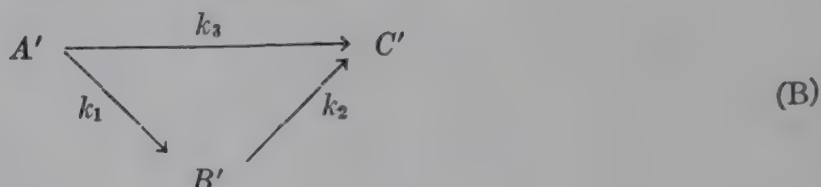


FIG. 6. The expected plot of the first order function, given two consecutive reactions.

than that value necessary for the reaction to be about 25 per cent complete. This statement is illustrated by Fig. 6, in which the right side of equation (5) is plotted *versus* time for various values of R . The portions of the lines for which the segments are nearly straight and parallel to the limiting line, $R = \infty$, define the circumstances under which equation (6) is an adequate approximation to equation (5). The right scale of the ordinate gives the extent of the reaction as calculated from the left scale of the ordinate. Ex-

trapolation of the straight segments of the lines to the axis of the ordinate gives non-zero intercepts, which correspond to the second term on the right side of equation (6). The numerical value of the intercept is large for small values of R . Thus given mechanism (A), linearity of the first order function, and a non-zero intercept, the value of R may be calculated.

To discuss the hydrolysis of labile phosphate from nucleic acid, the effect of alternative pathways must be considered.



For $k_1 + k_3 \neq k_2$

$$B = \frac{k_1 A_0}{k_2 - k_1 - k_3} [e^{-(k_1+k_3)t} - e^{-k_2 t}]$$

and

$$C = A_0 \left[1 - \frac{k_2 - k_3}{k_2 - k_1 - k_3} e^{-(k_1+k_3)t} + \frac{k_1}{k_2 - k_1 - k_3} e^{-k_2 t} \right]$$

A particularly interesting case is that in which $k_1 = k_2 = k_3$, for then C would be exactly equal to $A_0(1 - e^{-k_2 t})$, a first order expression. Since such a mechanism can be substituted for the step corresponding to k_2 in mechanism (B) without any effect on the first order function of the initial reactant and the final product, then it is clear that it is possible to have an indefinite number of equally slow reactions; yet the formation of the final product would follow an exact first order law. Thus it is impossible to draw any conclusions with respect to the number of slow reaction steps from the experimental facts which have been presented.

Additional information is provided by the fact that no experimental difference is obtained between the hydrolysis of labile phosphate and the hydrolysis of adenylic acid. The average value of d , taken from the nucleic acids, is 3.08, while that from the adenylic acid series is 3.10. The t^* values of three preparations of ribonucleic acid and dialyzable fragments were obtained by a special experimental procedure and will be discussed later. The average value of t^* for the other nucleic acids is 4.6 minutes, while that from the mixtures of adenylic and cytidylic acids is 4.8 minutes. These facts are contrary to the belief of Schmidt, Cubiles, and Thannhauser (2), that the induction period observed by them for the labile phosphate of nucleic acid corresponded to the hydrolysis of secondary phosphate bonds.

In order to identify the intermediate, the hydrolysis of which is the rate-determining step for both adenylic acid and labile phosphate, consider the

hydrolysis of adenylic acid in terms of mechanism (B). Substance *B'* is ribose-3-phosphate. From a consideration of the available information on the acidic hydrolysis of nucleotides (1, 10, 13, 16-22), a scheme for the correlation of the data was developed. The pertinent point is that the high lability of ribose-3-phosphate is due to the tendency of ribose to form furfural in hot mineral acid. Furfural formation can only occur if there is no ribosidic bond, for then the furanose ring can open, with subsequent closure between carbon atoms 2 and 5. The loss of phosphate is associated with the process of dehydration.

The purine nucleoside-3'-phosphates and the dihydropyrimidine nucleoside-3'-phosphates have extremely labile riboside bonds; thus the rates of phosphate hydrolysis equal that of ribose-3-phosphate. The pyrimidine nucleoside-3'-phosphates have stable riboside bonds and also stable phosphate bonds. Similar considerations apply to nucleotides of ribose-5-phosphate with the difference that ribose-5-phosphate is more stable than ribose-3-phosphate, presumably because ring closure between the 2 and 5 carbons is blocked.

It is known that the purine nucleoside-3'-phosphates and half of the ribose of nucleic acid yield furfural quantitatively, whereas the pyrimidine nucleotides yield very little furfural (23). Furthermore the rate of furfural formation from pentose (24), ribose-3-phosphate, and from compounds readily yielding ribose-3-phosphate is proportional to the concentration of acid. Pyrimidine nucleotides have rates more or less independent of the concentration of acid (16, 18). In particular, it has been determined in this laboratory that the rate of hydrolysis of adenylic acid in 1.0 *N* hydrochloric acid is twice the rate obtained in 0.5 *N* acid. The rate for cytidylic acid is only 5 per cent greater in 1.0 *N* acid than in 0.5 *N* acid.

It may be pointed out that hydrochloric acid and sulfuric acid are different in their catalytic abilities. An excellent example is the comparison of the two most extensive collections of hydrolytic data in this field, namely, the work of Jones and his collaborators and the data of this paper.

The striking property of ribose-3-phosphate is its high lability compared with other alcoholic phosphonates. The inference that ribose-3-phosphate is the intermediate permits the decision that the rate-determining step in the hydrolysis of labile phosphate occurs at the end of the sequence of reactions. Consequently mechanism (A) can be applied in that k_1 applies to the net rate of formation of free ribose-3-phosphate and k_2 applies to its rate of hydrolysis.

Before the ratio of the two k values can be calculated from equation (6), the temperature lag in the reaction vessels must be eliminated. A hot glycerol bath was used to bring the temperature of the reaction mixtures quickly to 100°. From the data obtained with three materials, indicated

in Table I, it is very probable that the true, isothermal t^* , hereafter indicated as t_0^* , is not greater than 1.5 minutes. From equation (6)

$$t_0^* = \frac{1}{k_2} \ln \left[\frac{k_1}{k_1 - k_2} \right]$$

from which

$$\frac{k_1}{k_2} \geq 13 \text{ and } k_1 \geq 42$$

The observations are compatible with the suggestion of Levene and Tipson (25) that phosphate in nucleic acid is cross-linked between positions 2 and 3 of adjacent ribose groups in nucleic acid, if the secondary phosphate bonds are very labile, and if esterification at the two position does not interfere with the hydrolysis of phosphate from the 3 position. The inferred rapid formation of free ribose phosphate might be incorrect if esterification at positions 2 and 5 does not interfere with the hydrolysis of phosphate from position 3. The dinucleotide diuridine-2':2'-phosphate was synthesized by Gulland and Smith (26) and the diester bond was found to be stable. Experiments are being designed to test the theory further.

The partitions of phosphate in the nucleic acids will be presented along with other analyses in another publication.

SUMMARY

1. The rates of hydrolysis of phosphate from the following substances in 0.5 N hydrochloric acid at 100° were studied: nucleic acids from yeast and from the pancreas, certain fragments obtained from the nucleic acid of yeast by the action of ribonuclease, and the four mononucleotides obtained from the nucleic acid of yeast.

2. The initial rate for the stable phosphate of nucleic acid is not significantly different from the average of the initial, but slightly different, rates which were obtained with the pyrimidine nucleotides.

3. No difference between the kinetics of hydrolysis of labile phosphate of nucleic acid and the kinetics of adenosine-3'-phosphate was detected.

4. Preparations of guanylic acid, although impure, give approximately the same hydrolysis rate as is obtained with adenylic acid.

5. It is postulated that free ribose-3-phosphate is the intermediate, the hydrolysis of which is the rate-determining step in the hydrolysis of labile phosphate from nucleic acid.

6. A simple procedure is described for the accurate determination of the partition of phosphate in nucleic acids.

BIBLIOGRAPHY

1. Jones, W., *Am. J. Physiol.*, **52**, 193 (1920).
2. Schmidt, G., Cubiles, R., and Thannhauser, S. J., *Cold Spring Harbor Symposia, Quant. Biol.*, **12**, 161 (1947).

3. Kobayashi, Y., *J. Biochem.*, Japan, **15**, 261 (1932).
4. Jorpes, E., *Acta med. Scand.*, **68**, 503 (1928).
5. Gulland, J. M., and Walsh, E. O., *J. Chem. Soc.*, 172 (1945).
6. Loring, H. S., Ordway, G. L., Roll, P. M., and Pierce, J. G., *Federation Proc.*, **6**, 510 (1947).
7. Loring, H. S., and Fairley, J. L., Jr., *J. Biol. Chem.*, **172**, 843 (1948).
8. Loring, H. S., Ordway, G. L., and Pierce, J. G., *J. Biol. Chem.*, **176**, 1123 (1948).
9. Vischer, E., and Chargaff, E., *J. Biol. Chem.*, **176**, 715 (1948).
10. Levene, P. A., and Bass, L. W., Nucleic acids, American Chemical Society monograph series, New York (1931).
11. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
12. Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **106**, 113 (1934).
13. Jones, W., and Read, B. E., *J. Biol. Chem.*, **31**, 337 (1917).
14. Gulland, J. M., and Jackson, E. M., *J. Chem. Soc.*, 1842 (1939).
15. Yamagawa, M., *J. Biol. Chem.*, **43**, 339 (1920).
16. Ishikawa, H., *J. Biochem.*, Japan, **22**, 385 (1935).
17. Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **121**, 131 (1937).
18. Levene, P. A., and Jorpes, E., *J. Biol. Chem.*, **81**, 575 (1929).
19. Embden, G., and Schmidt, G., *Z. physiol. Chem.*, **181**, 130 (1929); **197**, 191 (1931).
20. Albaum, H. G., and Umbreit, W. W., *J. Biol. Chem.*, **167**, 369 (1947).
21. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis, 164 (1945).
22. Gulland, J. M., and Smith, H., *J. Chem. Soc.*, 338 (1947).
23. Hoffman, W. S., *J. Biol. Chem.*, **73**, 15 (1927).
24. Dunlap, A. P., *Ind. and Eng. Chem.*, **40**, 204 (1948).
25. Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **109**, 623 (1935).
26. Gulland, J. M., and Smith, H., *J. Chem. Soc.*, 1532 (1948).

ISOTOPIC STUDIES OF PORPHYRIN AND HEMOGLOBIN METABOLISM

I. BIOSYNTHESIS OF COPROPORPHYRIN I AND ITS RELATIONSHIP TO HEMOGLOBIN METABOLISM*

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Coproporphyrins I and III have been identified in plant and animal tissues, but comparatively little is known about their biologic significance and their relation to hemoglobin protoporphyrin. Abnormally large amounts of coproporphyrin I are excreted both by patients with porphyria (particularly of the light-sensitive type) and by those with accelerated rates of erythropoiesis. Acute porphyria, chemical poisoning, some types of infections, and alcoholic cirrhosis are associated with increased excretion of coproporphyrin III (1, 2). Coproporphyrins I and III can be synthesized by certain unicellular organisms, *i.e.* yeast cells (3, 4) and *Corynebacterium diphtheriae* (5, 6); composition of the culture medium as well as the type of organism has been shown to influence the synthesis.

A new and promising approach to the study of porphyrin metabolism is provided by the recent demonstrations: (1) that the nitrogen (7) and α -carbon (8) of glycine are direct precursors of hemoglobin protoporphyrin, and (2) that hemoglobin protoporphyrin can consequently be tagged by feeding animals glycine labeled either with N^{15} or with C^{13} or C^{14} in the α position. If one assumes that the pyrrole nucleus is the primary unit involved in the biosynthesis of porphyrins, then other pyrrole compounds formed during the synthesis of protoporphyrin should contain these same isotopic precursors. Similarly, the degradation of hemoglobin containing tagged protoporphyrin should give rise to pyrrole compounds also labeled with N^{15} , C^{13} , or C^{14} .

The above concept forms the basis of the method used in these studies

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† Special Research Fellow of the National Institutes of Health.

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of porphyrin and hemoglobin metabolism. The present paper deals particularly with the biosynthesis of coproporphyrin I in dogs.

EXPERIMENTAL

Duplicate experiments were done in healthy mongrel dogs. The first part of each experiment (that involving Dogs I and III) was designed to confirm the demonstration that glycine nitrogen is incorporated into hemoglobin protoporphyrin, and to determine whether coproporphyrin I and stercobilin are formed as by-products in the synthesis of hemoglobin protoporphyrin. In the last half of each experiment, red blood cells with hemoglobin containing protoporphyrin tagged with N^{15} were transfused into animals (Dogs II and IV); consequently, in the recipient dog the increased excess N^{15} was present only in the hemoglobin. Hemolysis was then induced in order to determine whether excreted coproporphyrin I, protoporphyrin IX, and stercobilin are derived from hemoglobin breakdown. Protocols are given below; results are tabulated in Table I.

Experiment 1—Dog I, weight 12 kilos, was bled at intervals for 6 days to reduce its erythrocyte volume from 54 to 24 ml. of packed cells per 100 ml. of blood. At the time of the greatest anemia, 5.6 gm. of glycine containing 27.7 atom per cent excess N^{15} were given by stomach tube in six divided doses over a 3 day period. While the glycine was being administered, no food was allowed; thereafter, the animal was fed Purina dog chow *ad libitum*. On the 12th and 21st days following the first dose of glycine, 10 ml. samples of blood were obtained for the isolation of protoporphyrin IX dimethyl ester (9) and globin (10). Porphyrins were isolated from the urine and feces collected during the first 21 days. The initial extractions were made according to Dobriner's method (11); final purification and isolation were accomplished by chromatography on a $CaCO_3$ column with crystallization (12).

By the 34th day, the packed red cell volume had returned to 51 per cent. Blood was obtained for the isolation of hemoglobin protoporphyrin and for the determination of free erythrocyte protoporphyrin (free EP) (13). An ordinary transfusion set containing acid citrate-dextrose solution was then connected through an 18 gage needle to one of the femoral arteries, and the animal was bled to death. The collected cells, equivalent to 350 ml. of packed cells, were transfused into Dog II.

Dog II, weight 10 kilos, was made acutely anemic by two bleedings, 5 hours apart, of 250 ml. each. Immediately after the second hemorrhage, the animal was transfused with the cells from Dog I. The following day, a 10 ml. sample of blood was obtained; thus the N^{15} content of isolated crystalline protoporphyrin IX dimethyl ester could be determined. Phenylhydrazine anemia was then induced by the oral administration

of 0.8 gm. of phenylhydrazine hydrochloride in divided daily doses of 0.1 gm. over a 12 day period. The packed red cell volume decreased from 55 to 22.5 per cent on the 12th day. On the 21st day after the transfusion (cell volume 34 per cent, reticulocytes 15 per cent), blood was again drawn for the preparation of crystalline protoporphyrin IX dimethyl ester. From

TABLE I

*Atom Per Cent Excess N¹⁵ in Various Porphyrins (and Globin) during Synthesis and Breakdown of Hemoglobin**

Experiment No.	Blood			Urine and feces			
	Time in days after	Hb protoporphyrin IX dimethyl ester	Globin	Specimens collected	Coproporphyrin I tetramethyl ester	Protoporphyrin IX dimethyl ester	Stercobilin
1. Dog I	N ¹⁵ -Glycine 12	0.960	0.086	0-21 days after glycine	1.10		
	" " 21	0.780					
	" " 34	0.720					
Dog II	Transfusion 1	0.40		0-20 days after phenylhydrazine	0.03	0.133	
	Phenylhydrazine 20	0.132					
2. Dog III	N ¹⁵ -Glycine 12	0.710	0.094	0-12 days after glycine	0.960		0.760
	" " 24			13-24 days after glycine			0.253
	" " 36	0.490		0-15 days after phenylhydrazine	0.023	0.103	0.208
Dog IV	Transfusion 1	0.320					
	Phenylhydrazine 15	0.058					

* No coproporphyrin III or deuteroporphyrin IX could be isolated from any of the dogs with present methods.

the urine and feces quantitatively collected during the first 20 days, porphyrins were also isolated by the methods mentioned above.

Experiment 2—Dog III, weight 10 kilos, was bled repeatedly for 9 days. The cell volume decreased from 45 to 26 per cent, and the reticulocytes rose to 14 per cent. 5.15 gm. of glycine containing 31.9 atom per cent excess N¹⁵ were then given by stomach tube in six divided doses during a 3 day period. No food was allowed during these 3 days; a meat-free diet was fed thereafter. Crystalline protoporphyrin IX dimethyl ester

and globin were prepared from samples of blood drawn on the 12th and 36th days. Urine and feces were collected separately during the first 24 days in two equal periods of 12 days each. Feces were extracted (14) for the simultaneous isolation of porphyrins and stercobilin; final purification and isolation of the porphyrins were then accomplished (12). On the 36th day, the animal was bled to death. It had not completely recovered from the induced anemia and the red cells had the characteristics of a hypochromic anemia: red blood cells, 6,200,000 per cmm.; Hb, 9.8 gm. per 100 ml.; cell volume, 34.2 per cent; mean corpuscular hemoglobin concentration, 29 per cent; free EP, 120 γ per 100 ml. of packed red blood cells. The blood collected was centrifuged and the plasma was removed. The remaining packed cells, 296 ml., were transfused into Dog IV.

Dog IV weighed 11 kilos. After an acute anemia had been induced by the withdrawal of 500 ml. of blood in two bleedings separated by an interval of approximately 5 hours, the packed red blood cells from Dog III were injected intravenously. On the following day, the first of seven daily 0.1 gm. doses of phenylhydrazine hydrochloride was given by mouth. By the end of this period, the red cell volume had fallen from 57 to 20 per cent. Porphyrins and stercobilin were isolated from the urine and feces collected during the first 15 days following the first dose of phenylhydrazine. Blood was also collected on the 15th day for the preparation of crystalline protoporphyrin IX dimethyl ester. N^{15} concentrations in the various samples described in the above protocols were determined by the mass spectrometer.¹

Comments

The results of these experiments indicate that glycine serves in dogs as a direct nitrogenous precursor for coproporphyrin I and stercobilin, as well as for hemoglobin protoporphyrin (Table I). The fact that the N^{15} concentration of hemoglobin protoporphyrin in Dogs I and III was greater than that previously reported for rats (15) and humans (16) was probably due to the increased rate of hemoglobin formation stimulated by the bleedings. The subsequent decrease which occurred in the N^{15} concentration was probably caused by dilution with untagged hemoglobin formed during recovery from the induced anemia.

The high N^{15} concentration of the coproporphyrin excreted by Dogs I and III, at a time when hemoglobin (protoporphyrin IX) was being regenerated rapidly, indicates that coproporphyrin I is probably a product

¹ Determinations of the N^{15} concentration in samples from Dogs III and IV were made in Dr. Alfred O. C. Nier's laboratory, Department of Physics, University of Minnesota. We also wish to record our indebtedness to Dr. H. S. Anker, Department of Biochemistry, University of Chicago, whose cooperation made possible the isotopic analysis of samples from Dogs I and II.

of the same biosynthesis and is derived, most likely, from a common pyrrole precursor. Conversely, the very small N^{15} concentration of the coproporphyrin isolated from Dogs II and IV during periods of great hemolysis indicates that this porphyrin is not a product of hemoglobin breakdown. The small amount of N^{15} found probably resulted from the uptake from a "metabolic pool" introduced with the transfused cells.

The increased N^{15} concentration of stercobilin in Dog III during rapid hemoglobin synthesis confirms the observations of London *et al.* (17) in humans, and probably means that some stercobilin is formed from a source other than the degradation of circulating hemoglobin.

Finally, the increased N^{15} concentration of the protoporphyrin IX excreted by Dogs II and IV, at a time when hemolysis was greatly accelerated, suggests that at least a portion of fecal protoporphyrin may be derived from hemoglobin breakdown. The possibility exists, however, that it may have come from free EP in tagged, hemolyzed cells. The total amount of free EP transfused into Dogs III and IV was 268 and 356 γ respectively. The N^{15} concentration of the free EP fraction was not determined; thus this possibility cannot be excluded.

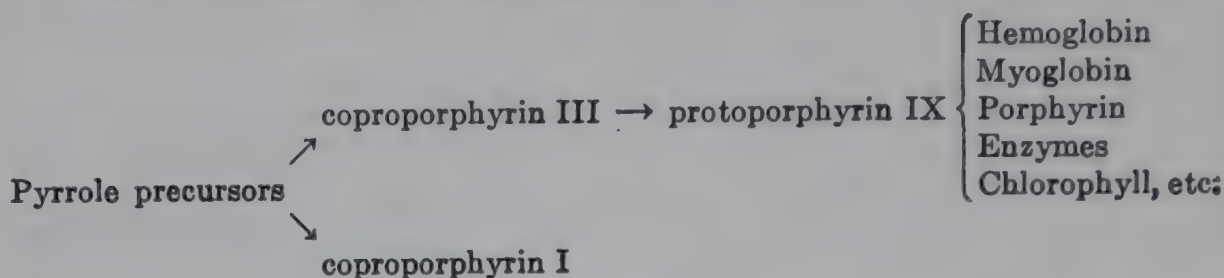
Rimington (18) and Dobriner and Rhoads (19) have postulated that coproporphyrin I is a by-product of the synthesis of a Type III porphyrin. The results reported are in accord with that hypothesis. The chemical nature of this Type III porphyrin is not known, although ultimately it becomes protoporphyrin IX. The *in vitro* synthesis of coproporphyrin III by the method of Fischer and Hierneis (20) leads to a mixture of coproporphyrin I and III. The same phenomenon may well take place in nature, and the ratio of the isomers may be regulated by an enzyme mechanism as suggested by Rimington (18). In that case, coproporphyrin III would be an intermediate metabolite, a precursor of protoporphyrin IX. There is no experimental evidence which excludes this possibility, and the following observations tend to support it:

1. An increase in coproporphyrin III excretion is frequently associated with a disturbance of hemoglobin metabolism, as in metal intoxication, sulfonamide therapy, etc. These increases may well be explained by blockage of the transformation of coproporphyrin III to protoporphyrin IX, or by a decrease in the utilization of protoporphyrin IX for hemoglobin metabolism. The concept that the reverse is true, that protoporphyrin IX is converted to coproporphyrin III, has not been supported by several investigators (21-23).

2. The work of Kench and Wilkinson (4), of Pappenheimer (5), and Rawlison and Hale (24) with yeast cells and *Corynebacterium diphtheriae*, respectively, suggests that coproporphyrin III is incorporated into enzymes. Thus far, the only porphyrin identified in enzymes is protoporphyrin IX.

If Granick's conclusion that protoporphyrin IX is a precursor of chloro-

phyll (25) is correct, one could explain the presence of the coproporphyrins in plants according to the following scheme.



SUMMARY

1. Glycine is a specific precursor, in dogs, of hemoglobin protoporphyrin, coproporphyrin I, and stercobilin.

2. Coproporphyrin I is formed during the biosynthesis of protoporphyrin IX and does not represent a hemoglobin derivative.

3. It is postulated that coproporphyrin I is a by-product of the synthesis of coproporphyrin III and that coproporphyrin III gives rise to protoporphyrin IX.

4. Protoporphyrin IX, excreted in the feces of dogs, is apparently a hemoglobin derivative.

5. Stercobilin is derived in part from sources other than circulating hemoglobin breakdown.

The authors desire to express their thanks to Dr. C. J. Watson for his interest and advice.

BIBLIOGRAPHY

1. Dobriner, K., and Rhoads, C. P., *Physiol. Rev.*, **20**, 416 (1940).
2. Watson, C. J., and Larson, E. A., *Physiol. Rev.*, **27**, 478 (1947).
3. Fischer, H., and Fink, H., *Z. physiol. Chem.*, **150**, 243 (1925).
4. Kench, J. E., and Wilkinson, J. F., *Nature*, **155**, 579 (1945); *Biochem. J.*, **40**, 660 (1946).
5. Pappenheimer, A. M., Jr., *J. Biol. Chem.*, **167**, 251 (1947).
6. Gray, C. H., and Halt, L. B., *Biochem. J.*, **43**, 191 (1948).
7. Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **159**, 567 (1945).
8. Altman, K. I., Casarett, G. W., Masters, R. E., Noonan, T. R., and Salomon, K., *J. Biol. Chem.*, **176**, 319 (1948).
9. Grinstein, M., *J. Biol. Chem.*, **167**, 515 (1947).
10. Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, **13**, 469 (1930).
11. Dobriner, K., *J. Biol. Chem.*, **120**, 115 (1937).
12. Grinstein, M., Schwartz, S., and Watson, C. J., *J. Biol. Chem.*, **157**, 323 (1945).
13. Grinstein, M., and Wintrobe, M. M., *J. Biol. Chem.*, **172**, 459 (1948).
14. Watson, C. J., *J. Biol. Chem.*, **105**, 469 (1934).
15. Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **166**, 621 (1946).
16. Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **166**, 627 (1946).
17. London, I., Shemin, D., and Rittenberg, D., *J. Clin. Invest.*, **27**, 547 (1948).

18. Rimington, C., *Compt.-rend. trav. Lab. Carlsberg, Série chim.*, **22**, 454 (1938).
19. Dobriner, K., and Rhoads, C. P., *J. Clin. Invest.*, **17**, 95 (1938).
20. Fischer, H., and Hierneis, J., *Z. physiol. Chem.*, **196**, 155 (1931).
21. Vigliani, E. C., *Arch. Sc. med.*, **65**, 391 (1938).
22. Salzburg, P., and Watson, C. J., *J. Biol. Chem.*, **139**, 593 (1941).
23. Watson, C. J., Pass, I. J., and Schwartz, S., *J. Biol. Chem.*, **139**, 583 (1941).
24. Rawlison, W. A., and Hale, J. H., *Biochem. J.*, **42**, p. xlvii (1948).
25. Granick, S., *J. Biol. Chem.*, **172**, 717 (1948); **175**, 333 (1948).

ISOTOPIC STUDIES OF PORPHYRIN AND HEMOGLOBIN METABOLISM

II. THE BIOSYNTHESIS OF COPROPORPHYRIN III IN EXPERIMENTAL LEAD POISONING*

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It is now well established that glycine is a direct nitrogenous precursor of hemoglobin protoporphyrin in rats (1), rabbits (2), dogs (3), and humans (4), of coproporphyrin I in dogs and humans (3, 5), of uroporphyrin I in humans (5), and of stercobilin in humans (5, 6). It is well known that lead intoxication in rabbits is associated with prompt and marked increases of urinary coproporphyrin III (7, 8). Three possibilities existed to explain these increases: (a) a derivation from destroyed hemoglobin (9), (b) new formation either in relation to a disturbed hemoglobin synthesis (10) or as an expression of some more obscure disturbance of pigment metabolism, (c) mobilization of free or bound coproporphyrin from tissue stores, with subsequent excretion in the urine.

The administration of glycine labelled with N^{15} to lead-poisoned rabbits offered a means of deciding which of these three possibilities is actually represented. The present paper describes the results of such a study.

Material and Methods

Rabbit 1, weighing 2.8 kilos, had received 200 mg. of lead acetate subcutaneously, as part of another study 6 months previously. The coproporphyrin III excretion was still high, 173 γ per day. 0.8 gm. of glycine containing 32 atom per cent excess N^{15} was administered by stomach tube in six doses over a 3 day period. On the 24th day thereafter, 10 ml. of blood were drawn and from this the protoporphyrin IX dimethyl ester was prepared in crystalline form, according to the usual method (11). The pooled urine for the entire 24 day period following the administration of glycine was subjected to the ether extraction-chromatography method of isolating coproporphyrin (12). The crystalline tetramethyl ester of coproporphyrin III was obtained for N^{15} analysis (see below).

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Rabbit 2, weight 2.4 kilos, received 100 mg. of lead acetate subcutaneously. On the 19th day, when the coproporphyrin III excretion had increased to 268 γ per day, 2.0 gm. of glycine containing 32 atom per cent excess N^{15} were administered intraperitoneally in fifteen doses and over a 3 day period. On the 10th and 29th days after the glycine had been injected, 10 ml. samples of blood were drawn and from each the protoporphyrin IX dimethyl ester was prepared in crystalline form.

The urine from Rabbit 2 was collected for two periods, *i.e.*, the first 9 days and the subsequent 18 days following the glycine administration. Coproporphyrin III tetramethyl ester was crystallized from the pooled urine of each period. The entire feces for 22 days were extracted in the usual manner (11) with acetic acid and ether, and the porphyrin was re-

TABLE I
Atom Per Cent Excess N^{15} of Various Porphyrins Isolated

Rabbit No.	Days after glycine administration	Hemoglobin protoporphyrin	Coproporphyrin III	Feces porphyrin x
1	24	0.066	0.076	
2	9		1.390	
	10	0.474		
	22			0.684
	27		0.107	
	29	0.214		
3	6		2.340	
	10	0.511		
	11			
	14			0.585
	21		0.097	0.147

moved from the washed ether with 5 per cent HCl (prepared in the proportion of 5 ml. of concentrated or 37 per cent HCl diluted with distilled H_2O to 37 ml.). The acid solution was made red to Congo paper by addition of sodium acetate, and the porphyrin reextracted with ether. It was then returned to a small volume of 5 per cent HCl and esterified by addition of several volumes of methyl alcohol saturated in the cold with HCl gas. The methyl ester was taken into chloroform and purified in the usual manner (11), after which it was chromatographed on a $CaCO_3$ column from a mixture of benzene-petroleum ether (1:1) and developed with benzene. The chromatogram showed two porphyrin zones, the upper of which was much larger. This porphyrin, as yet unidentified, will be referred to again as porphyrin x . The lower porphyrin zone on the column was identified as protoporphyrin IX.

The experiment in Rabbit 3, weighing 3.13 kilos, differed only in that

this animal received ultraviolet irradiation on a 10×10 cm. shaved area of skin for 20 minutes on the 1st, 6th, and 14th days following the administration of 2 gm. of glycine containing 32 atom per cent excess N^{15} . The reason for the radiation was the recent finding of Pimenta de Mello that ultraviolet radiation greatly increases the coproporphyrinuria of lead-poisoned rabbits.¹ Rabbit 3, in fact, showed a considerably greater increase of coproporphyrin in the urine than Rabbits 1 or 2. On the 10th day following the glycine a 10 ml. blood sample was drawn and the crystalline protoporphyrin methyl ester prepared. Urine and feces were collected for three consecutive periods of 1 week each for isolation of porphyrins as already mentioned.

The N^{15} concentration of the various porphyrins isolated was determined by the mass spectrometer.² The data are given in Table I.

Comments

The results obtained indicate that the glycine nitrogen is incorporated directly into the coproporphyrin III as well as into the hemoglobin protoporphyrin. The early drop in the N^{15} concentration of the hemoglobin protoporphyrin of Rabbit 2 is probably explained by a relatively more rapid hemoglobin destruction, due to the more acute lead poisoning in this animal. It is interesting to note that this drop is not accompanied by an increase in N^{15} concentration of the coproporphyrin III as one would expect if the latter were derived from hemoglobin protoporphyrin. The high concentration of N^{15} at 6 days after the glycine administration in Rabbit 3 speaks against a preformation, storage, and mobilization as the mechanism by which the excessive urinary coproporphyrin is explained. This leaves only the second of the three possibilities mentioned at the outset as a tenable concept, *i.e.*, a new formation of coproporphyrin III *pari passu* with a hemoglobin synthesis which may be disturbed, or as an expression of some more obscure disturbance of pigment metabolism. The general trend of the values is in accord with but does not prove a close relationship to hemoglobin synthesis. The possibility of an entirely separate formation of the coproporphyrin III, even quite apart from sites of erythropoiesis, cannot be excluded.

The data obtained with respect to the fecal porphyrin x are insufficient to permit conclusions regarding its nature and possible significance. Its chloroform solubility and general behavior, and its association with protoporphyrin, point to a possible relationship analogous, perhaps, to the relationship of the pseudodeute oporphyrins of human feces to proto-

¹ To be published.

² These determinations were carried out in Dr. Alfred O. C. Nier's laboratory, Department of Physics, University of Minnesota.

porphyrin. It is noteworthy that Fischer and Duesberg (8) described a porphyrin isolated from rabbit feces, similar to porphyrin x but differing in melting point of the methyl esters. Their porphyrin melted at 182–187°; the present porphyrin x at 210–215°. In so far as the N^{15} concentration noted in Table I is concerned, it is at least evident that the porphyrin x was not exogenous, since the glycine N^{15} was clearly utilized in its synthesis.

SUMMARY

Glycine is a direct nitrogenous precursor of the coproporphyrin III excreted in excess in the urine of lead-poisoned rabbits. The present data confirm the belief that this coproporphyrin III is not related to hemoglobin catabolism, and indicate that it is newly formed as a result of the lead poisoning, rather than a preformed porphyrin which had been stored and mobilized.

BIBLIOGRAPHY

1. Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **166**, 621 (1946).
2. Muir, H. M., and Neuberger, A., *Biochem. J.*, **40**, 43 (1948).
3. Grinstein, M., Kamen, M. D., and Moore, C. V., *J. Lab. and Clin. Med.*, **33**, 1478 (1948).
4. Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **159**, 567 (1945).
5. Grinstein, M., Aldrich, R., Hawkinson, V., and Watson, C. J., *J. Biol. Chem.*, **179**, 983 (1949).
6. London, I., Shemin, D., and Rittenberg, D., *J. Clin. Invest.*, **27**, 547 (1948).
7. Grotepass, W., *Z. physiol. Chem.*, **205**, 193 (1932).
8. Fischer, H., and Duesberg, R., *Arch. exp. Path. u. Pharmacol.*, **166**, 95 (1932).
9. Schreus, H. T., *Klin. Wochschr.*, **13**, 121 (1934).
10. Rimington, C., *Compt.-rend. trav. Lab. Carlsberg, Série chim.*, **22**, 454 (1938).
11. Grinstein, M., *J. Biol. Chem.*, **167**, 515 (1947).
12. Grinstein, M., Schwartz, S., and Watson, C. J., *J. Biol. Chem.*, **157**, 323 (1945).

STUDIES OF UNIDENTIFIED CHICK GROWTH FACTORS*

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Several investigators (1-6) have presented evidence that an unidentified factor, or factors, associated chiefly with animal protein materials, is required for optimum growth in the chick. The occurrence of this unidentified factor in various preparations from liver has been demonstrated (1, 3, 4, 7, 8). Moreover, a concentrate prepared from dried cow manure has been shown to be as effective in this respect as certain animal protein materials (9). Hill (10) obtained evidence for the multiple nature of the nutritional deficiency in all-vegetable protein chick diets. Ott *et al.* (11) reported that crystalline vitamin B₁₂ produces a growth response in chicks on certain diets low in the "animal protein factor." This has been confirmed by Lillie, Denton, and Bird (12). Nichol *et al.* (13) obtained evidence that crystalline vitamin B₁₂ can replace the animal protein factor activity of condensed fish solubles or parenteral liver extracts in counteracting the thyrotoxic conditions produced in chicks by feeding a basal diet containing iodinated casein.

The present investigation was undertaken for the purpose of developing concentrates of the unidentified factor, or factors, required by the chick when fed experimental diets in which all, or almost all, of the protein is of vegetable origin. The work was undertaken before the isolation of crystalline vitamin B₁₂ was reported by Rickes *et al.* (14) and by Smith (15).

Experimental Procedure and Results

Single comb white Leghorn chicks of both sexes were used in all experiments. The experimental groups were housed in electrically heated batter-

* This work was undertaken in cooperation with the Office of Naval Research, Navy Department, Washington, D. C., and was aided by grants to Cornell University by the Nutrition Foundation, Inc., New York, the Cerophyl Laboratories, Inc., Kansas City, Missouri, The Cooperative G. L. F. Exchange, Ithaca, New York, and the Western Condensing Company, San Francisco, California. A preliminary report of the work was made at the Informal Poultry Nutrition Conference held at Detroit, April 18 1949, in connection with the annual meeting of the Federation of American Societies for Experimental Biology.

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ies with raised screen floors. Feed and water were supplied *ad libitum*. In all but three of the experiments, the progeny of hens housed on raised wire floors and fed a practical diet containing little or no animal protein were used. The chicks were fed the basal Diet A shown in Table I during a preliminary period of 1 to 3 weeks, after which they were distributed into uniform groups on the basis of body weight. The chicks were then individually wing-banded and weighed at weekly intervals during the

TABLE I
Composition of Basal Diets

Ingredient	Diet A	Diet B
	gm.	gm.
Ground yellow corn.....	32.0	26.0
Ground wheat.....	25.0	
Soy bean meal (expeller).....	35.0	70.0
Dried whey.....	3.0	
Dicalcium phosphate.....	1.3	2.0
Limestone.....	2.7	1.0
Iodized salt.....	0.5	1.0
Cod liver oil*.....	0.5	0.5
	mg.	mg.
Manganese sulfate.....	33.0	12.5
Riboflavin.....	0.35	0.15
Calcium pantothenate.....	1.1	
Choline chloride.....	154.0	
Niacinamide.....	1.76	
Biotin.....	0.01	
Thiamine.....	0.2	
Pyridoxine hydrochloride.....	0.35	
Inositol.....	64.0	
Folic acid.....	0.055	
2-Methylnaphtholquinone.....		9.5

* Containing 400 A. O. A. C. units of vitamin D and 2000 i.u. of vitamin A per gm.

experimental period. The materials used to supply the unidentified factors were mixed into the basal diet.

A 95 per cent alcohol-soluble liver preparation, designated liver paste by the manufacturer,¹ was used as the source of the unidentified growth factors in all studies. A water solution of the liver paste was allowed to dialyze through a large cellophane sausage tube against distilled water for 18 days at 10°. The distilled water was replaced every 3 days. The dialysate was concentrated to approximately the original volume.

¹ Wilson and Company, Chicago, Illinois.

For further refinement, the dialysate was diluted with distilled water to a solid content of approximately 2 per cent, and the pH adjusted to 5.0 with hydrochloric acid. The resulting solution was passed through an adsorption column containing a mixture of activated alumina and Decalso (2:1). The filtrate from this adsorption was then concentrated to a convenient volume under a vacuum. Glacial acetic acid was added to make 2 per cent of the total volume, and the solution was extracted three times with 0.5 volume of *p*-cresol by use of a separatory funnel. The *p*-cresol layers were combined and washed twice with equal portions of 2 per cent acetic acid solution, and the washings discarded each time.

TABLE II
Chick Growth Response to Various Liver Fractions

Experiment No.	Lot No.	Treatment	Liver paste equivalence	No. of chicks used		Average total gain during experimental period*	Average daily gain
				Started	Surviving		
			<i>per cent</i>			<i>gm.</i>	<i>gm.</i>
1	1	None		11	11	206	7.4
	2	Liver paste	0.11	11	11	334	11.9
	3	" "	0.22	11	11	323	11.5
	4	Crude liver paste dialysate	0.22	11	11	322	11.5
	5	" " " "	0.44	11	10	327	11.7
2	1	None		10	9	215	7.7
	2	Crude liver paste dialysate	0.10	9	8	285	10.2
	3	Refined liver paste dialysate	0.075	9	8	230	8.2
	4	" " " "	0.15	9	8	293	10.5
	5	" " " "	0.30	9	8	303	10.8

* The experimental period was of 4 weeks duration.

The *p*-cresol was removed from the solution by the addition of water and repeated extractions with ethyl ether. The water solution was then concentrated to a small volume under a vacuum.

The chick growth results obtained by feeding the crude and the refined liver paste dialysates are shown in Table II. The dialysates were fed at levels equivalent to the original amount of liver paste from which they were prepared. Both the average total gain in weight and the average daily gain during the 4 week experimental period are given. The results show that the unidentified factor (or factors) required by the chick was dialyzable and was retained in appreciable quantities after purification with activated alumina and *p*-cresol.

The method of counter-current distribution, described by Craig (16), was used for the further purification of the refined dialysate containing the

unidentified growth factor (or factors). A series of separatory funnels of 1 liter capacity was used as counter-current tubes in lieu of the special apparatus described by Craig (16). The distribution was effected by transferring the lower layer of the solution from each of the funnels to the adjacent separatory funnel on the right. In this manner, a number of plates or series of equilibria were performed so that the same distribution occurred as would have been effected if the special apparatus had been employed. After each transfer, the two liquid phases were shaken for 2 minutes in an automatic shaker which operated at the rate of 140 shakes

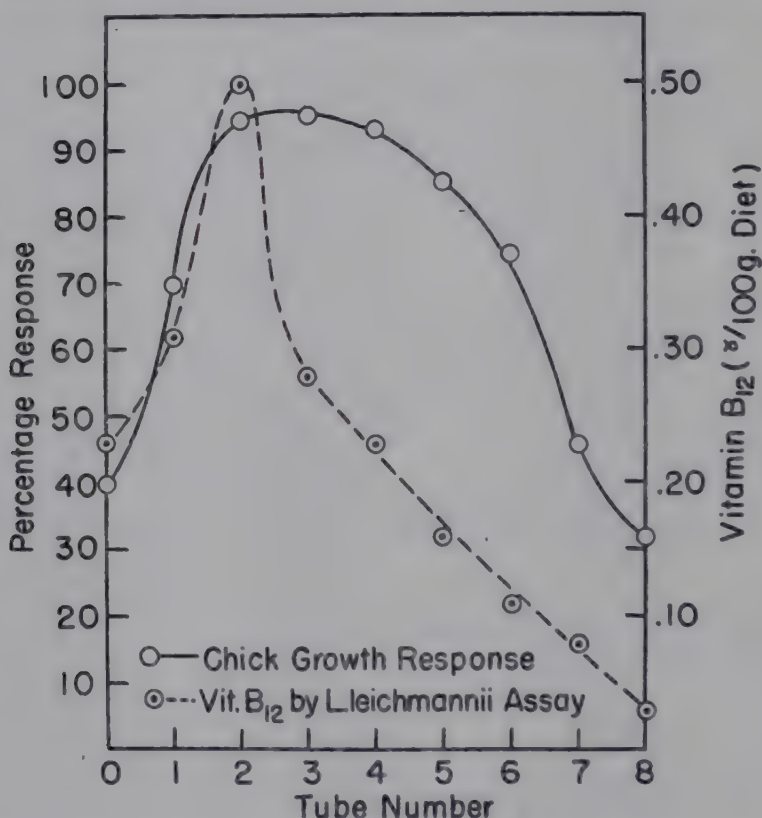


FIG. 1. Chick growth response and vitamin B₁₂ supplied with fractions obtained by an eight plate counter-current distribution of liver paste dialysate.

per minute. This period of shaking was found to be adequate to establish equilibrium.

A solvent system composed of an *n*-butyl alcohol layer containing 30 gm. of phenol per 100 ml. and a water layer consisting of 3 per cent glacial acetic acid and hydrochloric acid to bring the pH to 2.0 was used in the first counter-current distribution procedure. This involved eight plates. After the distribution was completed, the phenol and *n*-butyl alcohol were removed by repeated extraction with ethyl ether. The ether was removed by vacuum distillation with steam. The fractions distributed in the various tubes were fed to chicks at a level equivalent to 0.8 per cent of original liver paste. The results are presented graphically in

Fig. 1. For this purpose the percentage response of each lot was calculated by considering the difference between the negative and positive controls as 100 per cent. The results show that an unidentified chick growth factor (or factors) was located in Tubes 2, 3, 4, and 5.

Three superimposed counter-current distributions were then performed. The fractions in Tubes 2, 3, 4, and 5 from the first eight plate counter-current distribution were combined and subjected to a second eight plate counter-current distribution with the same solvent system. Finally, the fractions in Tubes 2, 3, 4, and 5 of the second counter-current distribution were combined, and a third counter-current distribution performed. This counter-current distribution procedure involved twelve plates and the solvent system was modified to contain 25 gm. of phenol per 100 ml. of *n*-butyl alcohol instead of the 30 gm. which were used in the first two eight plate counter-current distributions.

Before the material was subjected to the third counter-current distribution, it was extracted from a water solution by *p*-cresol in order to remove inorganic material. The fractions resulting from the final counter-current distributions were likewise extracted with *p*-cresol. Redistilled water was used in this procedure. The fractions which were obtained from Tubes 3 to 9 were then fed to chicks at a level of the diet equivalent to 0.9 per cent of original liver paste.

The fractionation by superimposed counter-current distribution and the chick studies were repeated, and the results of both studies combined. These are given in Fig. 2. Again, the chick response was expressed as percentage of maximum gain over the negative control.

From the data, it is evident that two peaks resulted in the growth response curve produced by feeding the fractions obtained from the different counter-current tubes. The peaks appeared to be located in Tube 5 and Tube 9 or 10. The chick growth response peaks found at two places in the series of counter-current distribution tubes indicated that at least two compounds, differing slightly in solubility in the two-phase liquid system, were involved in this study.

Later, an improved microbiological assay for vitamin B₁₂ (17) with *Lactobacillus leichmannii* ATCC 4797 was developed. Pure crystalline vitamin B₁₂² was used as a reference in this procedure. Upon assaying the material in all of the tubes of the initial eight plate counter-current distribution, vitamin B₁₂ was found to be concentrated in Tube 2, and the resulting curve more nearly represented a typical counter-current distribution curve than that obtained from the chick growth data. The microbiological curve is also presented in Fig. 1. When the material in all the

² Kindly supplied by Dr. E. Lester Smith of the Glaxo Laboratories Ltd., London, England.

tubes of the third superimposed counter-current distribution was assayed, evidence was obtained of the existence of two substances to which *L. leichmannii* responds. A curve showing the responses to the fractions in these various tubes is presented in Fig. 2.

Maximum responses obtained with *L. leichmannii* agree closely with maximum responses obtained in the chick studies, but the types of curves are quite different. Since paper partition chromatography failed to reveal the presence of thymidine in the refined liver paste dialysate, it is

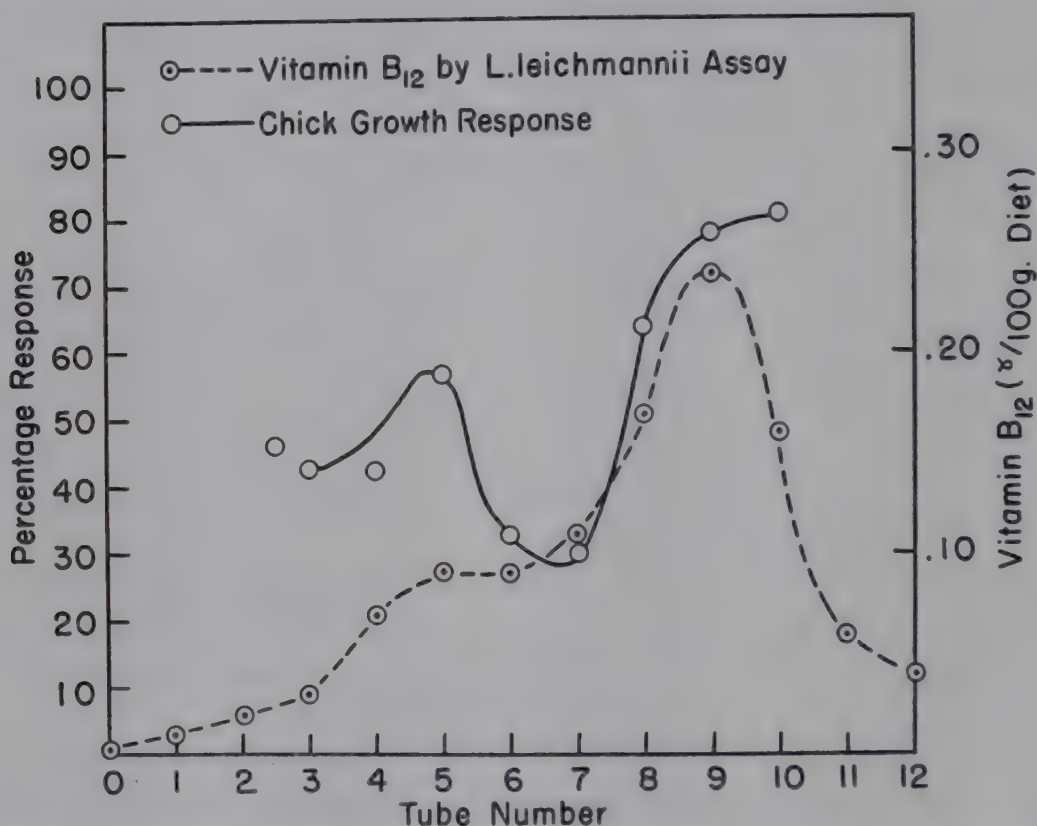


FIG. 2. Mean chick growth response and vitamin B₁₂ supplied with fractions from two twelve plate superimposed counter-current distributions of liver paste dialysate.

believed that the growth factors brought to light by the microbiological studies represent two different forms of vitamin B₁₂. The difference in the shapes of the two curves appears to be due, however, to the fact that the chick responded to one, and possibly two factors, in addition to the two forms of vitamin B₁₂, since only 0.03 γ of vitamin B₁₂ per 100 gm. of diet supplied by Tube 3 produced a highly significant growth response over the negative control group of chicks. The difference cannot be due to the fact that the chick shows a greater response to the form of vitamin B₁₂ concentrated in Tube 5 than does *L. leichmannii*. The additive effect would give a curve similar to the microbiological response curve of the two vitamin B₁₂ forms rather than the chick response curve with its characteristic dip at Tube 7.

At this stage in the investigation it was no longer possible to use chicks from depleted hens as the experimental subjects, and resort was made to hatchery single comb white Leghorn male chicks from hens fed a normal diet. In order to make these chicks as responsive to supplements of the unidentified factors as chicks from depleted hens, a procedure described by Rubin and Bird (18) was employed. Chicks were fed Diet B (Table I) in which the protein content was increased from approximately 20 per cent to approximately 33 per cent by raising the amount of soy bean meal in the diet to 70 per cent. The chicks were fed this basal diet for 3 weeks

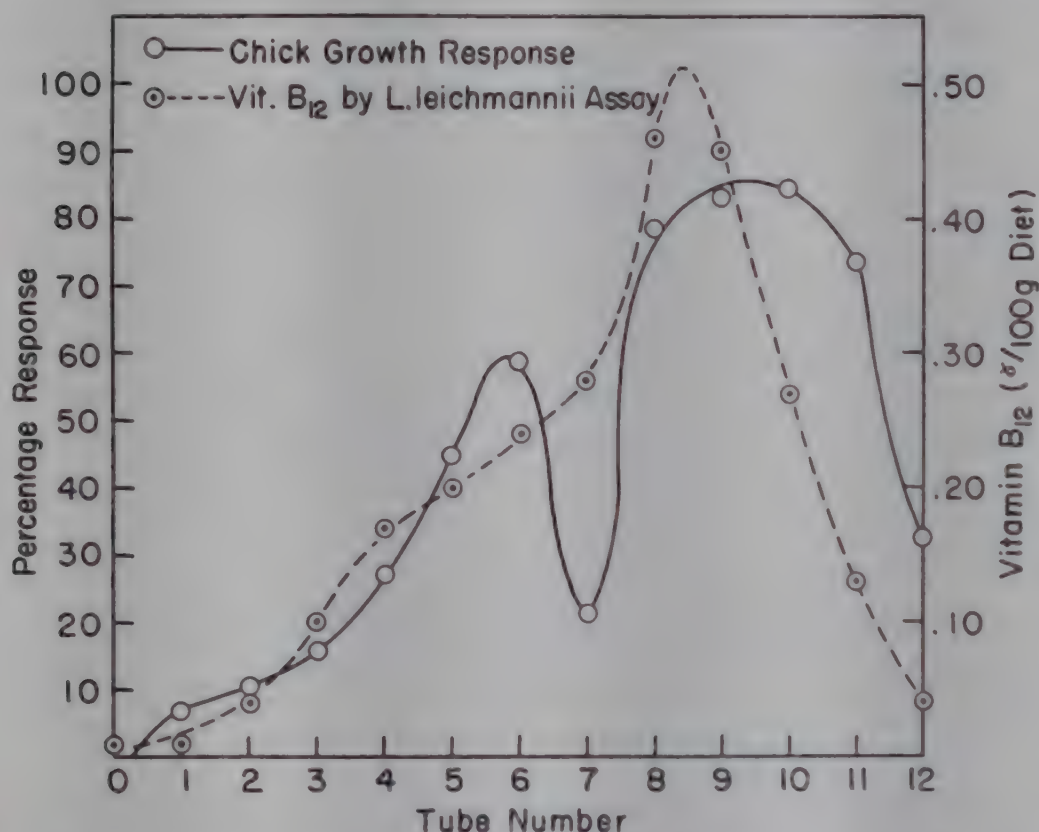


FIG. 3. Mean chick growth response and vitamin B₁₂ supplied with fractions from two twelve plate counter-current distributions of liver paste dialysate.

after hatching in order to deplete them of their reserves of the unidentified factors. A depletion period as long as this is possible owing to the fact that the chicks were obtained from non-depleted hens.

Two experiments were conducted with fractions obtained by an initial twelve plate counter-current distribution, prepared in the manner previously described. The amount of phenol per 100 ml. of *n*-butyl alcohol was 25 gm. The fractions from each tube were fed separately along with a negative control lot and a lot receiving crude liver paste dialysate as a positive control. All fractions were fed at levels equivalent to 0.6 per cent of liver paste.

The results obtained in these two experiments are presented graphically in Fig. 3. They confirmed the results obtained previously showing the

presence of two chick growth factors in the fractions obtained by the counter-current distribution procedure. Peaks in the growth response curve were obtained at Tube 6 and at Tubes 9 and 10. The peak at Tubes 9 and 10, however, is broad, which is indicative of the response expected when an optimum amount of two necessary growth factors is provided in a diet deficient in both factors and is not typical of a counter-current distribution curve. The growth obtained at the peak was not equal to that of the positive control, nor was maximum growth obtained at the other peak, but the shape of the curve was more typical of a counter-current distribution curve.

Upon assaying the fractions obtained by an initial twelve plate counter-current distribution with *L. leichmannii*, a response curve was obtained which was almost identical with that obtained previously by assaying the fractions from the three superimposed counter-current distributions. The response curve is given in Fig. 3. When this curve is compared with the chick response curve, it becomes evident that the chicks again responded to the fractions in Tubes 4, 5, and 6 in a manner not in accord with their vitamin B₁₂ activity and to Tubes 11 and 12 which contained little or no vitamin B₁₂. The growth of the chicks fed the material in the latter tubes, however, was significantly greater than that of the negative control. According to the results of the microbiological assay, the amount of vitamin B₁₂ supplied to the chicks fed the material in Tubes 11 and 12 was 0.13 γ and 0.04 γ respectively per 100 gm. of diet. These levels in the form of pure vitamin B₁₂ are insufficient to have any material growth-promoting effect on chicks. It appears, therefore, that two factors, or two forms of the same factor, in addition to the two forms of vitamin B₁₂, are deficient in Diet B.

The material in the tubes obtained in the twelve plate counter-current distribution was also assayed for factors other than vitamin B₁₂ with *L. leichmannii* according to the method described by Peeler and associates (17) and with *Lactobacillus casei* in accordance with the procedure reported by Daniel *et al.* (19). The response curves are presented in Fig. 4. The results indicate that the presence of an unidentified factor required by the microorganisms in Tubes 10, 11, and 12 is correlated with the response of the chicks to an unidentified factor in these tubes. Whether or not the two factors are identical cannot be determined from the evidence. The possibility suggests itself, however, that these microorganisms may be used in the development of microbiological assays for the unidentified chick factor.

In an effort to obtain further evidence of the existence of this factor, an experiment was conducted in which graded levels of the material in Tubes 10, 11, and 12 were fed. At the time the experiment was conducted, the

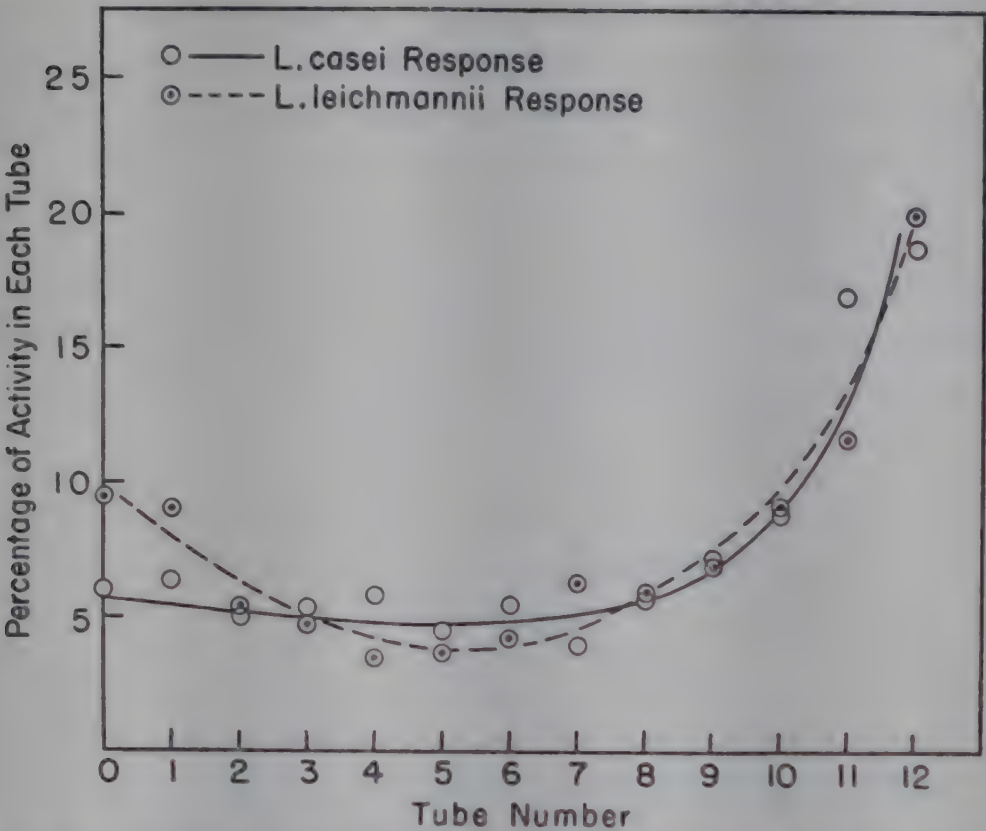


FIG. 4. Microbiological response to an unknown factor in fractions obtained by a twelve plate counter-current distribution of liver paste dialysate.

TABLE III
Chick Growth Response to Graded Levels of Various Liver Fractions

Supplement	Liver paste equivalence	Average total gain*	Percentage response†	Vitamin B ₁₂ (L. leichmannii assay)
	per cent	gm.		γ per 100 gm.
None.....		94		
Tubes 4-6.....	0.1	121	10.5	0.10
“ 4-6.....	0.2	173	30.7	0.20
“ 4-6.....	0.3	233	54.1	0.30
“ 10-12.....	0.1	255	62.6	0.07
“ 10-12.....	0.2	250	60.7	0.14
“ 10-12.....	0.3	285	74.3	0.21
“ 4-6, 10-12.....	0.1	286	74.7	0.17
“ 4-6, 10-12.....	0.2	308	83.3	0.34
Liver paste dialysate.....	0.05	269	68.1	0.17
“ “ “.....	0.10	346	98.1	0.35
“ “ “.....	0.20	351	100.0	0.70

* The experimental period was of 4 weeks duration.
† Per cent of maximum gain over negative control.

improved microbiological assay with *L. leichmannii* had not been developed. Therefore, for a source of the other factor, graded levels of Tubes 4, 5, and 6 were fed. Graded levels of crude liver paste dialysate were fed as positive controls. Two combinations of the material in these two sets of tubes were also fed in order to determine whether any supplementary relationship existed. The results of this experiment are presented in Table III, in which a column is included, giving the amount of vitamin B₁₂, determined later by microbiological assay, supplied the chicks in the various lots in addition to that already present in the basal diet.

The results indicate that a plateau was obtained by feeding graded levels of the fractions from Tubes 10, 11, and 12. The increase in growth obtained by feeding a level equivalent to 0.1 per cent of liver paste over the negative control was highly significant, whereas the increase in growth obtained by tripling the level of this material was of no statistical significance.

A graded growth response was obtained by feeding graded levels of the material in Tubes 4, 5, and 6. The increase in growth obtained on the two higher levels of this material over the negative control was highly significant as was also the difference in growth response between these two levels.

Some evidence of a mutual supplementary effect was obtained by combining the materials from the two sets of tubes. This was only evident, however, in the combination providing the higher levels of these materials. At these levels the amount of vitamin B₁₂ provided was the same as that for the lot fed the highest level of the material in Tubes 4, 5, and 6. The increase in growth produced by the combination over that of the chicks fed this level of material in Tubes 4, 5, and 6 alone was, however, highly significant.

SUMMARY

Growth-stimulating factors in fractions obtained from refined liver paste dialysate by Craig's procedure of counter-current distribution have been studied by means of chick growth and microbiological assays. The results of these studies indicate the existence of four unidentified substances which promote rapid early growth in chicks. By the use of an improved microbiological assay with *Lactobacillus leichmannii*, evidence was obtained that two of these factors are different forms of vitamin B₁₂. The other two substances do not appear to be identical with vitamin B₁₂ or any of the known vitamins.

Significant growth responses have been obtained by feeding fractions containing either of these unknown factors at levels which supplied approximately 0.03 to 0.04 γ of vitamin B₁₂ per 100 gm. of diet. This level

of vitamin B₁₂ is insufficient to have any material growth-promoting effect on chicks. Some evidence of a mutual supplementary effect was also obtained by feeding a combination of fractions containing these two unknown factors.

In the presence of adequate vitamin B₁₂, *L. leichmannii* has been found to respond to an unknown factor obtained from counter-current distribution fractions. These fractions also contain one of the unknown chick growth factors. *Lactobacillus casei* has also been found to respond markedly to these fractions. Whether or not the chick and microbiological factors are identical has not been determined.

BIBLIOGRAPHY

1. Johnson, E. L., Carrick, C. W., Roberts, R. E., and Hauge, S. M., *Poultry Sc.*, **21**, 566 (1942).
2. Heuser, G. F., and Norris, L. C., *Cornell Univ. Agr. Exp. Sta., Bull.* 810 (1940).
3. Heuser, G. F., Norris, L. C., Lucas, H. L., and Combs, G. F., *Poultry Sc.*, **25**, 403 (1946).
4. McGinnis, J., Stevens, J. M., and Groves, K., *Poultry Sc.*, **26**, 550 (1947).
5. Robblee, A. R., Nichol, C. A., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., *Poultry Sc.*, **26**, 553 (1947).
6. Combs, G. F., Heuser, G. F., and Norris, L. C., *Poultry Sc.*, **27**, 238 (1948).
7. Berry, E. P., Carrick, C. W., Roberts, R. E., and Hauge, S. M., *Poultry Sc.*, **24**, 270 (1945).
8. Nichol, C. A., Robblee, A. R., Cravens, W. W., and Elvehjem, C. A., *J. Biol. Chem.*, **170**, 419 (1947).
9. Rubin, M., and Bird, H. R., *J. Biol. Chem.*, **163**, 393 (1946).
10. Hill, F. W., *Poultry Sc.*, **27**, 536 (1948).
11. Ott, W. H., Rickes, E. L., and Wood, T. R., *J. Biol. Chem.*, **174**, 1047 (1948).
12. Lillie, R. J., Denton, C. A., and Bird, H. R., *J. Biol. Chem.*, **176**, 1477 (1948).
13. Nichol, C. A., Dietrich, L. S., Cravens, W. W., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, **70**, 40 (1949).
14. Rickes, W. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K., *Science*, **107**, 396 (1948).
15. Smith, E. L., *Nature*, **161**, 638 (1948).
16. Craig, L. C., *J. Biol. Chem.*, **155**, 519 (1944).
17. Peeler, H. T., Yacowitz, H., and Norris, L. C., *Proc. Soc. Exp. Biol. and Med.*, **72**, 515 (1949).
18. Rubin, M., and Bird, H. R., *Federation Proc.*, **6**, 419 (1947).
19. Daniel, L. J., Peeler, H. T., Norris, L. C., and Scott, M. L., *J. Biol. Chem.*, **177**, 917 (1949).

ON THE MECHANISM OF ALLOXAN HYPOGLYCEMIA

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Intravenous injection of alloxan into rabbits, rats, dogs, monkeys, etc., produces a brief hyperglycemia, then a transient hypoglycemia, which in the case of rabbits is severe enough to cause convulsions and death, unless counteracted by repeated injections of glucose, and finally a permanent diabetic hyperglycemia.

The cause of the transient hypoglycemia has had various explanations. Goldner and Gomori (1, 2), Kennedy and Lukens (3), and Banerjee (4) consider that the secondary hypoglycemia is pancreatic in origin, due to the liberation of insulin by the β cells of the islets undergoing destruction. Houssay and his associates (5), however, attribute the hypoglycemia to an extrapancreatic effect, *viz.*, lack of glucose production by the liver.

Recently, Banerjee and Bhattacharya (6) observed no hypoglycemic convulsions in rabbits fasted and phlorhizinized for a period of 7 days and then injected with diabetogenic doses of alloxan. The present paper is an extension of this study of the mechanism of alloxan hypoglycemia. The effect of the injection of diabetogenic doses of alloxan on blood sugar levels has been investigated in rabbits with yellow phosphorus liver damage, with persistent phlorhizin glycosuria, and after fasting. A comparison has also been made of the glycogen content of livers of rabbits after periods of fasting and during convulsive seizures due to injections of diabetogenic doses of alloxan or due to excessive doses of insulin.

EXPERIMENTAL

Five healthy Himalayan rabbits, each approximately 2 kilos in body weight, were kept in separate metabolism cages. Liver damage was produced in all the animals by the oral administration of two to six doses of yellow phosphorus (7), each dose being 3 mg. per kilo. The phosphorus was given twice or three times per week as a 0.25 per cent solution in olive oil. Liver damage was indicated by a diabetic type of glucose tolerance curve (8). Glucose tolerance curves of three of the animals both before and after the administration of phosphorus are compared in Fig. 1. After liver damage had been established in the animals, alloxan in a dose of 200 mg. per kilo was injected into the marginal ear veins of all the animals and the blood sugar values were determined at varying intervals of time up to

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24 hours (Table I). Blood sugar was determined according to the method of Hagedorn and Jensen (9).

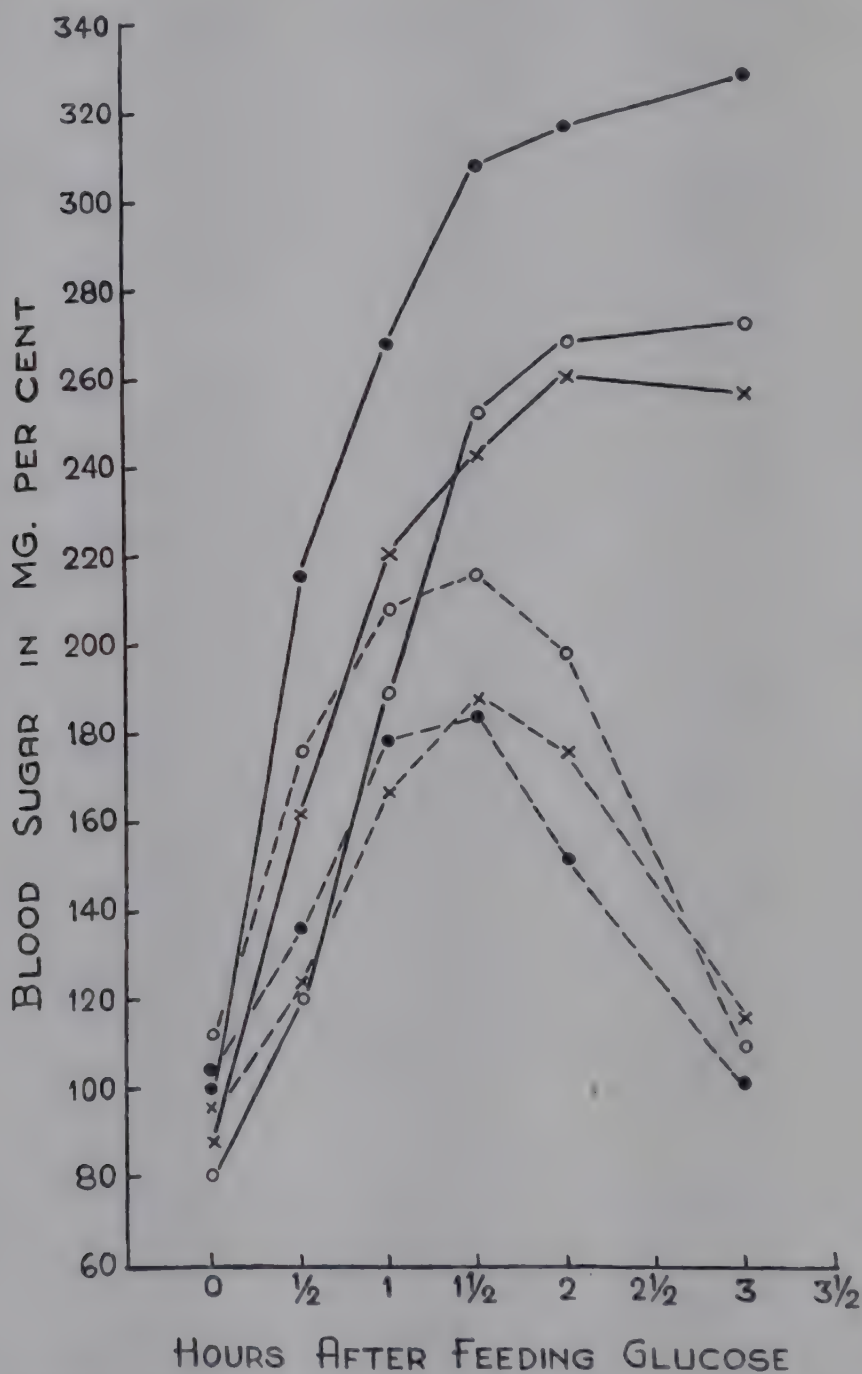


FIG. 1. Glucose tolerance curves of rabbits before (dash curves) and after (solid curves) liver injury was produced by the oral administration of yellow phosphorus. Glucose tolerance was determined by the oral administration of 2 gm. of glucose per kilo of body weight.

Another set of six rabbits, also kept in separate metabolism cages, was given seven daily injections of 100 mg. of phlorhizin suspended in olive oil. All the animals excreted sugar in the urine by the 2nd day of the experi-

ment. After seven doses of phlorhizin, glucose tolerance was determined in three of the animals (Fig. 2). Alloxan in diabetogenic doses was then injected intravenously into all the animals. Blood sugar was determined in samples taken both before and at varying intervals up to 24 hours after the injection of alloxan (Table II).

TABLE I

Effect of Injection of Diabetogenic Doses of Alloxan on Blood Sugar Levels in Rabbits with Livers Damaged with Yellow Phosphorus

Rabbit No.	Blood sugar after injection of alloxan, mg. per cent										
	0 hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	7 hrs.	8 hrs.	9 hrs.	24 hrs.
1	100	126	169	241	378	382	383	317	231	149	208
2	105	156		171	185		203	218	233	189	196
3	69		85	65	67		60	64	64	67	241
4	95	118	135	169	194	209		222	165	132	215
5	92	131		211	256	268	271	215	156	115	305

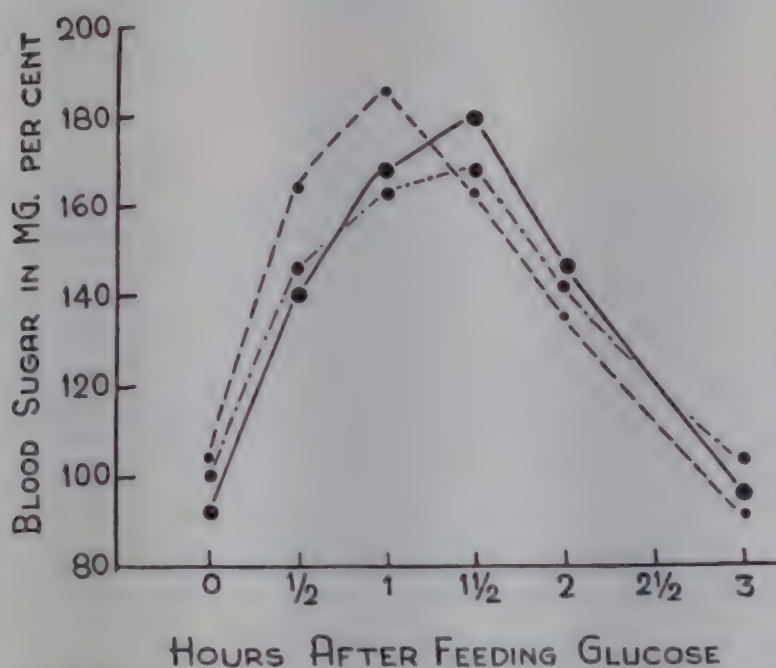


FIG. 2. Glucose tolerance curves of three rabbits phlorhizinized for 7 days. Glucose tolerance was determined by the oral administration of 2 gm. of glucose per kilo of body weight.

Three more rabbits were fasted for 7 days but were allowed to drink water during this period. Glucose tolerance was determined in these fasted animals, as well as the effect of the injection of diabetogenic doses of alloxan.

Nine rabbits were fasted overnight. On the following day alloxan in

diabetogenic doses (200 mg. per kilo) was injected into a group of three, so that the animals were in convulsions approximately 24 hours after food had been withdrawn. Insulin (10 units) was administered to a second group of three, so that these rabbits were also in convulsions approximately 24 hours after food had been withdrawn. The remaining three animals were simply fasted for the 24 hour period. All the animals were sacrificed by a sudden blow on the head. The throat was cut open, the liver rapidly removed, and a weighed portion was put into boiling 60 per cent potas-

TABLE II
Effect of Injection of Diabetogenic Doses of Alloxan on Blood Sugar Levels in Phlorhizinized Rabbits

Rabbit No.	Blood sugar after injection of alloxan, mg. per cent									
	0 hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	7 hrs.	8 hrs.	24 hrs.
1	87	122	167	167	153	85	74	72	69	196
2	100		124		116	103	91	87	82	260
3	95		128		117	87	79		75	210
4	105		285	240	155		95		84	305
5	92		129		131		93	88		338
6	98		143	134		98	89		78	326

TABLE III
Liver Glycogen Levels in Rabbits after 24 Hour Fasting Period, with and without Convulsions Induced by Alloxan and by Insulin

Three rabbits in each group.

Normal	During convulsions due to injection of	
	Diabetogenic doses of alloxan	10 units of insulin
	gm. per cent	gm. per cent
2.5	4.03	1.21
2.2	4.30	1.36
2.8	4.98	1.08

sium hydroxide. The glycogen was precipitated, hydrolyzed, and estimated according to the method of Evans, Tsai, and Young (10). The results are given in Table III.

Results

Rabbits with livers damaged with phosphorus showed a diabetic type of glucose tolerance curve with fasting blood sugar values almost normal (Fig. 1). Intravenous injection of diabetogenic doses of alloxan into four such rabbits produced an initial hyperglycemia but no hypoglycemia

(Table I). Rabbit 3 showed practically no fluctuation of blood sugar up to the 9th hour after the injection of alloxan. All of the rabbits showed high blood sugar and excreted sugar in the urine after 24 hours.

Rabbits phlorhizinized for 7 days gave glucose tolerance curves corresponding to those in normal animals (Fig. 2). Intravenous injection of diabetogenic doses of alloxan into such rabbits produced an initial hyperglycemia and a mild hypoglycemia 6 or 7 hours after the injection but never produced hypoglycemic convulsions (Table II). All of the animals survived the next day without injection of glucose and developed diabetes.

Rabbits fasted for 7 days gave the diabetic type of glucose tolerance curve, due possibly to a reduced insulin content of the pancreas (11). All of the fasted rabbits developed hypoglycemic convulsions within 4 to 6 hours after the intravenous injection of diabetogenic doses of alloxan.

The glycogen content of the livers of rabbits in convulsions due to the injection of diabetogenic doses of alloxan was found to be several times greater than that of livers of rabbits in convulsions due to the injection of insulin. The glycogen content of livers of normal rabbits fasted for 24 hours was lower than that of rabbits in convulsions due to the injection of alloxan but greater than that of rabbits in convulsions due to the injection of insulin (Table III).

DISCUSSION

Normal rabbits develop hypoglycemic convulsions within 4 to 6 hours after the intravenous injection of diabetogenic doses (200 mg. per kilo) of alloxan. None of the rabbits with livers damaged with yellow phosphorus developed hypoglycemia, even 9 hours after the injection of alloxan. All of the animals survived without injections of glucose and developed diabetes. Phosphorus damages liver by destroying parenchymatous tissues and hampers the process of glycogenesis (8). The process of glycogenolysis in the liver is consequently rendered more or less invalid, for in the absence of glycogenesis there is no scope for glycogenolysis. Animals with severe liver damage consequently exhibit a diabetic type of glucose tolerance, as was confirmed in our experiments.

Houssay *et al.* (5) are of the opinion that the secondary alloxan hypoglycemia is extrapancreatic in origin and that it is probably due to a temporary blockage of glycogenolysis in the liver. Others believe that the hypoglycemia is pancreatic in origin and that it results from the release of preformed insulin in the necrosed islets of the pancreas. In rabbits with livers damaged with phosphorus the process of hepatic glycogenolysis, as explained above, is rendered more or less invalid, but the pancreas is left intact, as evidenced by the approximately normal values for fasting blood sugar. Injection of alloxan into such rabbits should have produced hypo-

glycemia if its origin were pancreatic, for the β cells were destroyed as usual, as was demonstrated by the onset of diabetes after 24 hours. On the other hand, there should have been no hypoglycemia if it were hepatic in origin, due to a temporary blockage of glycogenolysis in the liver. The results obtained show that the cause of the secondary hypoglycemia is extrapancreatic and probably hepatic in origin.

The results in Table III show that insulin hypoglycemia and convulsions are associated with a decreased glycogen content of the liver, whereas alloxan hypoglycemia and convulsions are associated with an increased level of hepatic glycogen. This tends to show that the hypoglycemia and convulsions due to injections of insulin and alloxan, respectively, are probably of different origin. Insulin hypoglycemia is due to excessive utilization of glucose in the tissues leading to depletion of the glycogen content of the liver, whereas alloxan hypoglycemia is probably due to a blockage of glycogenolysis in the liver leading to the accumulation of extra glycogen.

Phlorhizinized animals have no dearth of insulin and the islets are not damaged in any way, as shown by the fact that the glucose tolerance curves are almost normal and by the fact that the omission of phlorhizin rapidly restores the animals to the normal state. The effect of phlorhizin is only to lower the renal threshold with resulting excretion of sugar in the urine. Loss of sugar in the urine, however, has the same effect on the liver as diabetes produced by pancreatectomy or administration of alloxan. Liver glycogenolysis compensates in part for the sugar lost and this leads to depletion of liver glycogen. Injection of alloxan in phlorhizinized rabbits destroyed the β cells as usual, but there was only limited hypoglycemia, if any. These results also agree with the hypothesis that the hypoglycemia is not pancreatic in origin, that the phenomenon is somehow associated with the process of glycogenolysis in the normal liver, and that it is probably due to a blockage of glycogenolysis. Alloxan cannot possibly check the process of glycogenolysis in livers in which the process has already been carried to an abnormal extreme, as in phlorhizinized animals.

Inhibition of hepatic glycogenolysis may explain the results obtained by Houssay *et al.* (5) who reported hypoglycemia by the injection of alloxan into dogs pancreatectomized half an hour earlier; that is, in dogs in which the livers were not fully diabetic. The same authors, however, could not produce hypoglycemia in dogs 24 hours after pancreatectomy, by which time the livers certainly were diabetic. This may also explain the results of Banerjee (4) and Goldner and Gomori (2) who observed that half pancreatectomized rabbits failed to develop hypoglycemic convulsions (although they developed moderate hypoglycemia) after the injection of alloxan. The results obtained by Banerjee and Bhattacharya (6) were possibly due not to the combined effect of fasting and of phlorhizin in re-

ducing the insulin content of the pancreas but to the effect of phlorhizin alone in rendering the liver more or less diabetic, as has been established in the present paper. Fasting alone led to impaired carbohydrate utilization, due possibly to the reduced insulin content of the pancreas. Fasting could not, however, lessen the severity of alloxan hypoglycemia.

SUMMARY

1. Intravenous injection of diabetogenic doses (200 mg. per kilo) of alloxan into rabbits, in which the livers were damaged by the oral administration of yellow phosphorus, did not produce hypoglycemia.

2. Intravenous injection of diabetogenic doses of alloxan into rabbits with persistent glycosuria due to daily administration of phlorhizin for 7 days also failed to produce any marked hypoglycemia.

3. Rabbits fasted for a period of 7 days developed hypoglycemia convulsions within 4 to 6 hours after the intravenous injection of diabetogenic doses of alloxan.

4. The glycogen content of the livers of rabbits is increased in alloxan hypoglycemia, whereas it is decreased in insulin hypoglycemia.

5. Alloxan hypoglycemia, as observed in rabbits, appears to be extra-pancreatic and is probably due to a temporary blockage of liver glycogenolysis.

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BIBLIOGRAPHY

1. Goldner, M. G., and Gomori, G., *Proc. Soc. Exp. Biol. and Med.*, **55**, 73 (1944).
2. Goldner, M. G., and Gomori, G., *Proc. Soc. Exp. Biol. and Med.*, **65**, 18 (1947).
3. Kennedy, W. B., and Lukens, F. D. W., *Proc. Soc. Exp. Biol. and Med.*, **57**, 143 (1944).
4. Banerjee, S., *J. Biol. Chem.*, **158**, 547 (1945).
5. Houssay, B. A., Orias, O., and Sara, T., *Science*, **102**, 197 (1945).
6. Banerjee, S., and Bhattacharya, G., *J. Biol. Chem.*, **175**, 923 (1948).
7. Deichmann, W. B., Kitzmiller, K. V., and Witherup, S., *Arch. Biochem.*, **7**, 409 (1945).
8. Peters, J. P., and Van Slyke, D. D., *Quantitative clinical chemistry, Interpretations*, Baltimore, 2nd edition, **1**, 257 (1946).
9. Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, **137**, 92 (1923).
10. Evans, C. L., Tsai, C., and Young, F. G., *J. Physiol.*, **73**, 67 (1931).
11. Best, C. H., Haist, R. E., and Ridout, J. H., *J. Physiol.*, **97**, 107 (1939).

THE RÔLE OF CARBAMYL-L-GLUTAMIC ACID IN THE ENZYMATIC SYNTHESIS OF CITRULLINE FROM ORNITHINE*

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It has been generally assumed that the first reaction in the Krebs urea cycle is the non-enzymatic carboxylation of ornithine to give δ -carbamino-ornithine which later combines with ammonia in a second reaction (1) resulting in the synthesis of citrulline. After the separation by differential centrifugation of the enzymatic system responsible for the formation of citrulline (2), it was possible to demonstrate that the synthesis from ornithine requires glutamic acid and adenosine triphosphate (ATP) in addition to carbon dioxide and ammonia.

The δ -carbaminoornithine \rightarrow citrulline reaction has never been demonstrated. Recently Leuthardt and Brunner (3) pointed out that a direct carboxylation of the δ -amino group of ornithine would be improbable in view of the high pK value of this group (10.76). Previously Leuthardt and Glasson (4) postulated that the synthesis of citrulline resulted from a coupling reaction with oxalacetic amide.

In an attempt to obtain evidence for the formation of δ -carbamino-ornithine, experiments were devised to test the possibility of a direct carbon dioxide fixation by ornithine. For this purpose a known amount of carbon dioxide was liberated inside specially constructed Warburg flasks after a preliminary equilibration period. Under various experimental conditions no difference in the rate of gas uptake could be detected in the presence or absence of washed liver preparations if an oxidizable substrate was absent. If glutamic acid was added to the system containing ornithine the total gas uptake was increased by an amount which paralleled the quantity of citrulline synthesized. This observation as well as the high specificity for glutamic acid in this system as demonstrated by Cohen and Hayano (5) led us to the more detailed study of the rôle of the glutamic acid in the reaction. We have reported previously that the formation of citrulline from ornithine most probably involves an intermediate glutamic acid derivative (6, 7). In this paper experiments bearing on the nature of this intermediate are presented.

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† In part, this study was carried out during the tenure of a fellowship from the Del Amo Foundation.

Procedures and Preparations

The washed rat liver preparation described by Cohen and Hayano (2) was used.

DL-Ornithine was obtained from the Amino Acid Manufactures at Los Angeles. In all experiments in which DL-ornithine was used, the concentration is indicated on the basis of the L isomer.

L-Glutamic acid decarboxylase (*Escherichia coli*) was a gift from Dr. R. H. Burris, Department of Biochemistry, University of Wisconsin.

ATP (adenosine triphosphate) was prepared according to the directions of LePage (8), AMP (adenosine monophosphate) was made from ATP by the method of Kerr (9), and DPN (diphosphopyridine nucleotide) was prepared according to Williamson and Green (10). TPN (triphosphopyridine nucleotide) was made according to Altman's modification¹ of the method of Warburg, Christian, and Griesche (11).

Cytochrome *c* was prepared by the method of Keilin and Hartree (12).

α -Ketoglutaric acid was synthesized according to Wisclicenus and Waldmuller (13) and Neuberg and Ringer (14).

Oxalacetic acid was prepared by the hydrolysis of ethyloxalacetic ester according to Simon (15).

cis-Aconitic acid was synthesized by the procedures outlined by Lardy (16).

D-Glutamic acid was a gift from Dr. J. P. Greenstein of the National Cancer Institute, Bethesda, Maryland.

Carbamyl-DL-aspartic acid, carbamyl-L-leucine, carbamyl-DL-alanine, benzoyl-DL-glutamic acid, and a sample of carbamyl-L-glutamic acid were gifts from Dr. D. G. Doherty, Department of Biochemistry, University of Wisconsin.

Carbamyl-L-glutamic acid (m.p. 160°), carbamyl-D-glutamic acid (m.p. 162–163°), α -carbamyl-DL-ornithine (m.p. 226°), 5-propionic acid hydantoin (m.p. 179–181°), and carbamylglycine (m.p. 168–169°) were prepared essentially according to the procedures of Nyc and Mitchell (17). The semicarbazone of α -ketoglutaric acid (m.p. 218°) and the oxime of α -ketoglutaric acid (m.p. 139°) were prepared in the usual manner. Phenyl-carbamyl-L-glutamic acid (m.p. 127–128°) was prepared by the procedure of Hopkins and Wormall (18); formyl-L-glutamic acid (m.p. 178–180°) by the procedure of Carter and West (19); and succinamic acid (m.p. 153–154°) by the hydrolysis of succinimide.

The γ -ethyl ester of L-glutamic acid was prepared according to Bergmann and Zervas (20). γ -Ethyl carbamyl-L-glutamic acid ester (m.p. 162–164°; N, calculated, 12.8 per cent, found, 12.7 per cent) was prepared

¹ Personal communication.

by treating the γ -ethyl ester of glutamic acid with KCNO. Carbamylglutamine (m.p. 158–160° with decomposition; N, calculated, 22.2 per cent; found, 21.9 per cent) was prepared by treating γ -ethyl carbamylglutamic acid ester with concentrated NH_3 for 3 days at room temperature. The solution was then acidified and treated with ethyl alcohol and ether in the cold. The resulting precipitate was recrystallized from hot alcoholic solution. Ninhydrin tests were negative for both compounds. Carbamylglutamine gave the delayed Nessler test typical of glutamine.

L- α -Guanidinoglutaric acid (decomposed at 206°) was prepared from L-glutamic acid essentially according to Wheeler and Merriam (21)² (N, calculated, 22.2 per cent; found, 23.4 per cent). The compound gave a positive Sakaguchi test. The high nitrogen content suggests that a portion of the material may have cyclized.

Urea was determined either by the Krebs and Henseleit manometric method (22) or by the Archibald colorimetric method (23).

Citrulline was determined according to Archibald (24).

The authors wish to acknowledge with thanks the generosity of the above donors for the compounds listed.

Results

Effect of Preincubation of Glutamic Acid, Ammonia, and Carbon Dioxide with Rat Liver Residue on Synthesis of Citrulline—It is clear from Fig. 1 that there is an increase in citrulline formation if the enzyme is incubated with glutamic acid, ammonia, and carbon dioxide prior to the addition of ornithine. This effect is noted for the first 30 minutes, but longer incubations do not increase the rate of synthesis with respect to controls. It should be pointed out that the incubation of ornithine with ammonia and carbon dioxide, prior to the addition of glutamic acid, does not increase the formation of citrulline. This finding excludes the direct reaction of the δ -amino group of ornithine with carbon dioxide to form a carbamino intermediate. It should be emphasized that there is no significant decrease in activity of the enzyme preparation kept in the side arm of the vessel in the absence of substrate under the above conditions. This finding will be discussed further under "Properties of enzyme system."

Effect of Enzyme Concentration at Different ATP Levels—The formation of citrulline in the presence of the optimal concentrations of substrates at three different levels of ATP and at varying dilutions of the tissue is shown in Fig. 2; a characteristic enzyme dilution effect is observed, which is independent of the conditions of incubation. In the preincubation

² The authors are indebted to Mr. Seymour Koritz of this department for the synthesis of γ -ethyl-L-glutamic acid ester, γ -ethylcarbamylglutamic acid ester, carbamylglutamine, and guanidinoglutaric acid.

experiments, the rate of citrulline synthesis is doubled when the concentration of ATP is doubled from 4.5×10^{-4} M to 9×10^{-4} M. Doubling of the latter concentration of ATP to 1.8×10^{-3} M results in an approximate doubling of citrulline synthesis only at the lower tissue concentrations. While increasing the ATP concentration in the experiments without previous incubation results in a marked increase in citrulline synthesis, the magnitude of the latter is never as great as that observed in the pre-

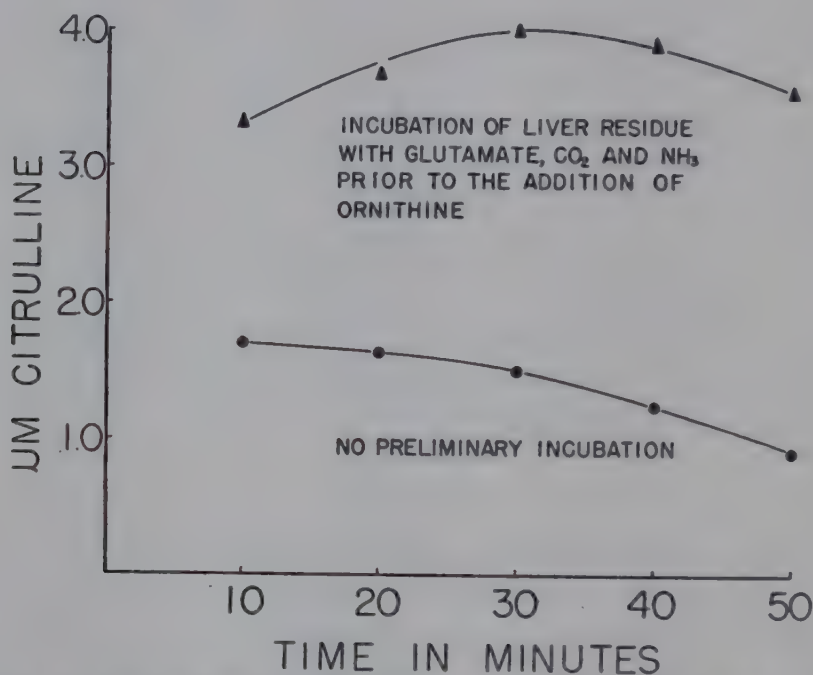


FIG. 1. Effect of preincubation of glutamate, ammonia, and bicarbonate ions with rat liver residue on the synthesis of citrulline. Final substrate concentrations, DL-ornithine, 2.5×10^{-3} M; magnesium sulfate, 6×10^{-3} M; ATP, 1.2×10^{-3} M; AMP, 1.2×10^{-3} M; potassium phosphate buffer at pH 7.15, 1.3×10^{-2} M; L-glutamate, 3.8×10^{-2} M; ammonium chloride, 4×10^{-3} M; bicarbonate ions, 5×10^{-3} M, and potassium ions to bring the medium to isotonicity. Tissue concentration, 2.8 mg. of N per flask. Final volume, 4.0 ml. Gas phase, air. Incubation of the whole system 12 minutes at 38° . The upper curve refers to experiments in which the enzyme system has been incubated with ammonia, bicarbonate ions, and glutamate prior to the addition of ornithine. The lower curve indicates the "blank" values obtained without preliminary incubation for the lengths of time indicated in the graph.

incubation experiments. At the lowest concentration of ATP used, 4.5×10^{-4} M, the amount of citrulline synthesized appears to run parallel with increasing tissue concentration in the two sets of experiments with and without a previous incubation period. The marked dilution effect in the case of the experiment with the lowest ATP concentration without a previous incubation period is striking.

Comparative Activity of ATP and AMP—As has been shown in earlier experiments by Cohen and Hayano (2), both ATP and AMP stimulate

the synthesis of citrulline. These results have been confirmed in the present experiments. In all the ranges tested, ATP and AMP showed

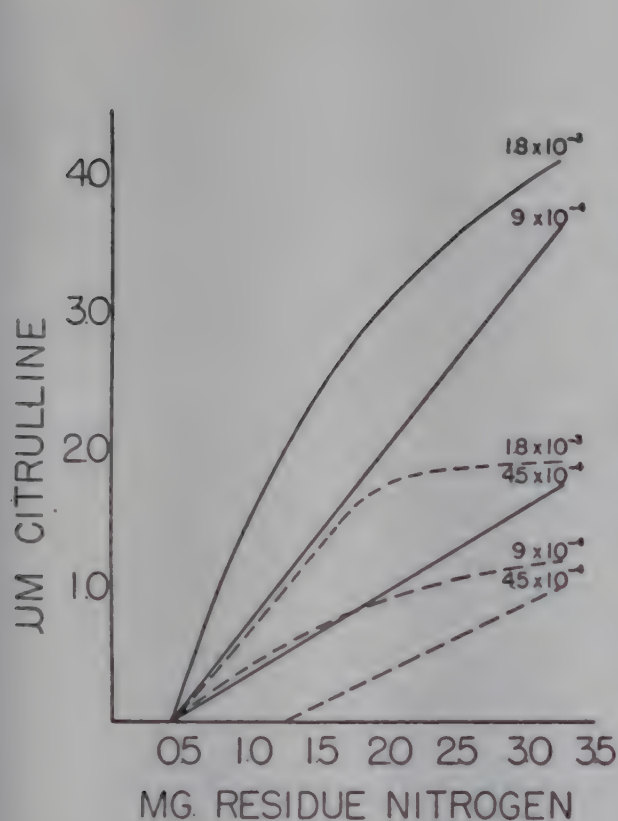


FIG. 2

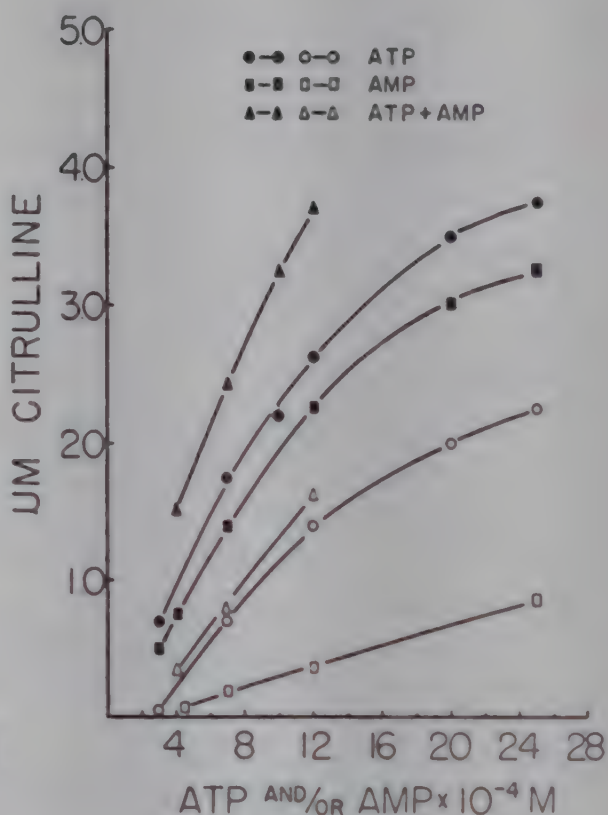


FIG. 3

FIG. 2. Effect of enzyme concentration at different ATP levels. Final substrate concentrations, magnesium sulfate, 6×10^{-3} M; potassium phosphate buffer at pH 7.15, 1.3×10^{-2} M; bicarbonate ions, 5×10^{-3} M; DL-ornithine, 2.5×10^{-3} M; ammonium chloride, 4×10^{-3} M; glutamate, 3.8×10^{-2} M. ATP concentrations (final molarity) indicated by the values along the curves. Final volume, 4.0 ml. Incubation time for the complete system, 10 minutes. Gas phase, air. Temperature 38° . The solid lines indicate incubation of the enzyme system for 20 minutes with ammonia, bicarbonate ions, and glutamate prior to the addition of ornithine. The dotted lines indicate the values obtained without preliminary incubation.

FIG. 3. Effect of increasing concentrations of AMP, ATP, or both on the formation of citrulline. Final substrate concentrations, DL-ornithine, 2.5×10^{-3} M; magnesium sulfate, 6×10^{-3} M; potassium phosphate buffer at pH 7.15, 1.3×10^{-2} M; L-glutamate, 3.8×10^{-2} M; ammonium chloride, 4×10^{-3} M; bicarbonate ions, 5×10^{-3} M, and potassium ions to bring the medium to isotonicity. Tissue concentration, 2.9 mg. of N per flask. Final volume, 4.0 ml. Gas phase, air. Incubation, 10 minutes at 38° . The solid symbols represent experiments in which the enzyme system has been previously incubated for 20 minutes with L-glutamate, ammonia, and bicarbonate ions. The open symbols represent the "blank" values obtained without such a preliminary incubation.

a very close relationship (Fig. 3). The effect of previous incubation is more striking in the case of AMP than in the case of ATP. This suggests that during the incubation period previous to ornithine addition AMP

is converted to ATP. When both ATP and AMP are present, the effect on synthesis of citrulline can be seen to be approximately additive.

Effect of Gas Phase—Under strictly anaerobic conditions there is no synthesis of citrulline by washed rat liver residue from glutamic acid, carbon dioxide, ammonia, and ornithine or from carbamylglutamic acid, ammonia, and ornithine. Previously, we suggested that the synthesis of citrulline could be divided into two steps (6). The first step was considered to be concerned with the aerobic formation of an intermediate which in turn participates in the second step which could proceed under anaerobic conditions. We have observed citrulline synthesis from carbamylglutamic acid under anaerobic conditions provided there has been previous aerobic incubation of the system before the addition of ornithine. While this effect has also been observed with glutamic acid, a considerably greater rate is observed with carbamylglutamic acid (see Figs. 4 and 5). Methylene blue, high fumaric concentrations, high ATP concentration, and ferricyanide do not support the synthesis of citrulline in a nitrogen atmosphere, either from glutamic acid or from carbamylglutamic acid. Attempts to support the synthesis by coupling the reaction with the α -ketoglutarate dismutation system to form glutamate (25) were unsuccessful.

Replacement of Glutamic Acid in Synthesis of Citrulline—As has been shown by Cohen and Hayano (5), glutamic acid seems to play a specific rôle in the formation of citrulline. We have attempted to replace glutamic acid by other metabolites, all other experimental conditions being kept constant. The results are summarized in Table I.

Glutamic acid and glutamine appear to be equally active at the concentrations studied. The considerable activity of α -ketoglutaric acid and proline is, in all probability, due to the ready conversion of these to glutamic acid (26–28). D-Glutamic acid and substituted glutamic acids are not active. Of the amino acids studied, glutamic acid alone has a high activity. The appreciable activity of the tricarboxylic acid cycle intermediates appears to be due to the formation of glutamic acid via α -ketoglutaric acid and ammonia.

The replacement of glutamate by other oxidizable substrates, as indicated in Table I, shows that citric acid is less effective than *cis*-aconitate and isocitrate. An indication that aconitase may be a limiting factor is the finding that the synthesis of citrulline in the presence of isocitrate approximates the sum of that formed for citrate and *cis*-aconitate. No adequate explanation is at hand for the greater efficiency of fumarate in this system when compared with the other members of the tricarboxylic cycle.

Carbamyl Group Precursors—The possible rôle of compounds other than

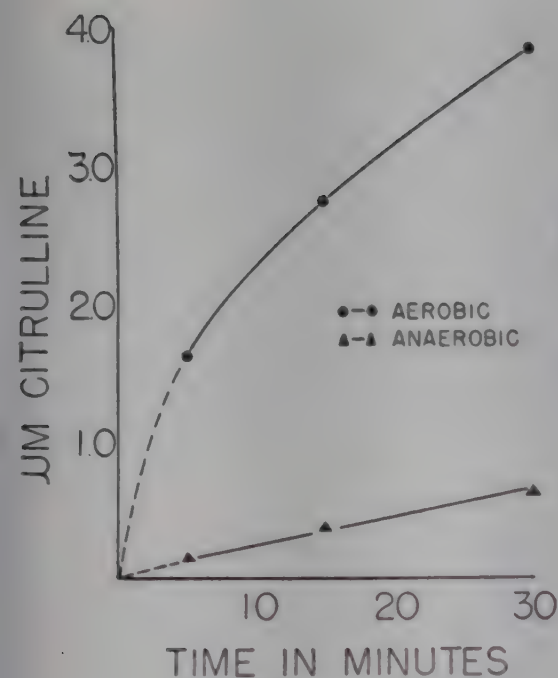


FIG. 4

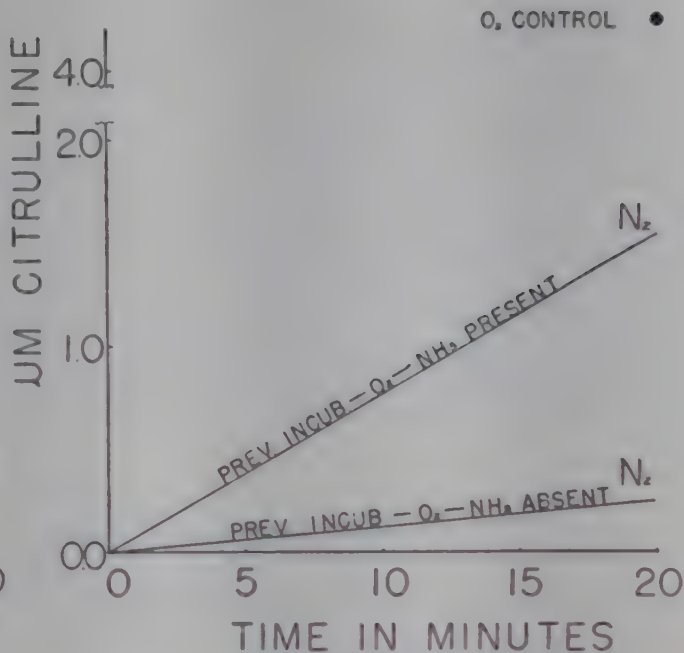


FIG. 5

FIG. 4. Comparison of aerobic and anaerobic incubation after different times of previous aerobic incubation of the enzyme system with L-glutamate, ammonia, and bicarbonate ions. Final substrate concentrations, magnesium sulfate, 6×10^{-3} M; ATP, 1.2×10^{-3} M; AMP, 1.2×10^{-3} M; potassium phosphate buffer at pH 7.15, 1.3×10^{-2} M; ammonium chloride, 4×10^{-3} M; bicarbonate ions, 5×10^{-3} M; DL-ornithine, 2.5×10^{-3} M; L-glutamate, 3.8×10^{-2} M, and potassium ions to bring the medium to isotonicity. Tissue concentrations 3.0 mg. of N per flask. Final volume, 4.0 ml. Temperature, 38° . Time of incubation for the complete system, 10 minutes, after the previous aerobic incubation of the enzyme system with L-glutamate, bicarbonate ions, and ammonia for the time intervals indicated on the abscissa. The upper curve represents the values obtained by the aerobic incubation of the complete system. The lower curve represents the values for the anaerobic incubation of the complete system after the previous aerobic incubation for the times indicated on the graph.

FIG. 5. Comparison of aerobic and anaerobic incubation after different previous aerobic incubation periods of the enzyme system with carbamyl-L-glutamate and carbamyl-L-glutamate plus ammonia. Final substrate concentrations, magnesium sulfate, 6×10^{-3} M; potassium phosphate buffer at pH 7.15, 1.3×10^{-2} M; fumarate, 2×10^{-2} M; ammonium chloride, 2×10^{-3} M; carbamyl-L-glutamate, 3.8×10^{-3} M; DL-ornithine, 2.5×10^{-3} M; ATP, 1.2×10^{-3} M; AMP, 4×10^{-4} M, and potassium ions to bring the medium to isotonicity. Tissue concentration, 4.5 mg. Temperature 38° . Final volume, 4.0 ml. Gas phase as indicated. Time of incubation for the complete system, 30 minutes, after the previous incubation of the enzyme system with carbamyl-L-glutamate for the time intervals indicated on the abscissa. The upper dot represents the aerobic incubation for the complete system. The upper line represents the values for the anaerobic incubation of the complete system after the previous aerobic incubation of carbamyl-L-glutamate with ammonia and the enzyme system in presence of Mg, K and phosphate ions, ATP, and AMP. The lower curve represents the same experiment, except that ammonia has been excluded during the aerobic incubation.

carbon dioxide and ammonia as immediate precursors of the carbamyl group was investigated. Experimental conditions were the same as those described in Table I. Molar equivalents of ammonia and bicarbonate were used in the control experiments. The results are summarized in Table II. It is apparent that none of the compounds studied was as effective as ammonia and carbon dioxide. It is also evident that amide groups are not directly involved in the carbamylation reaction but only indirectly to the extent that they give rise to carbon dioxide and ammonia. The relatively high activity of formic acid plus ammonia appears to be due to the oxidation of formic acid to carbon dioxide. The possibility that

TABLE I
Citrulline Synthesis with Compounds Other Than Glutamic Acid

Relative rates expressed as per cent of values obtained with L-glutamate for same incubation period			
L-Glutamate	100	Isocitrate	12.0
L-Glutamine	100	L-Hydroxyproline	8.5
α -Ketoglutarate	66.2	Citrate*	8.25
L-Proline	40.2	Citrate	6.3
Oxalacetate	36.0	L-Leucine	2.2
Fumarate*	35.0	L-Aspartate	1.2
Pyruvate	32.0	L-Alanine	0.0
Fumarate	30.0	Benzoyl-L-glutamate	0.0
Succinate	24.0	D-Glutamate	0.0
Isocitrate*	21.2	Glutathione	0.0
cis-Aconitate*	12.2		

Final substrate concentrations, magnesium sulfate, 6×10^{-3} M; ATP, 1.2×10^{-3} M; AMP, 1.2×10^{-3} M; potassium phosphate buffer at pH 7.15, 1.3×10^{-2} M; DL-ornithine, 2.5×10^{-3} M; ammonium chloride, 4×10^{-3} M; bicarbonate ions, 5×10^{-3} M, and potassium ions to bring the medium to isotonicity. All compounds listed were present at 3.8×10^{-2} M. Tissue concentration, 2.7 mg. of N per flask. Final volume, 4.0 ml. Gas phase, air. 10 minutes incubation at 38°.

* Incubation for 40 minutes.

formamide might be involved in the carbamylation reaction appears to be ruled out from the present experiments. The semicarbazide and oxime of α -ketoglutaric acid are not active in this system.

Effect of Carbamyl and Related Compounds on Synthesis of Citrulline—The experimental data reported above led to the study of compounds which might be expected to play a specific rôle in the reaction. Carbamyl-L-glutamic acid was the most active of the compounds examined so far (Table III). It is possible to demonstrate this effect not only in carbon dioxide-free systems (7) but also in the presence of carbon dioxide. Under the latter conditions, carbamyl-L-glutamic acid is 2 to 3 times more active

than L-glutamic acid. Substitution in the carbamyl group by a phenyl group results in a complete loss of activity of the carbamyl compound. Carbamyl amino acids other than L-glutamic show little or no activity. It should be emphasized that, in order to obtain maximum effects with carbamylglutamic acid, it is required that the medium used be freshly prepared, freed of carbon dioxide in a vacuum at pH 5.0, and neutralized to pH 7.15 immediately before use. The low activity observed with carbamylglutamine was unexpected, since it was considered likely, for reasons discussed later, that this compound might be more active than carbamylglutamic acid. It is rather surprising that it is not at least as

TABLE II
Carbamyl Group Precursors

With glutamate		Without glutamate	
Ammonia + CO ₂	100	α -Ketoglutarate semicarbazide + fumarate	2
Formate + ammonia	50	Formamide	0
Formamide	25	Formate + ammonia	0
Succinamate	23	α -Ketoglutarate oxime + fumarate	0
Lactamide	20		
Leucine + CO ₂	20		
Asparagine + CO ₂	10		

Final substrate concentrations, magnesium sulfate, 6×10^{-3} M; ATP, 1.2×10^{-3} M; AMP, 1.2×10^{-3} M; potassium phosphate buffer at pH 7.15, 1.3×10^{-2} M; DL-ornithine, 2.5×10^{-3} M; ammonium chloride, 4×10^{-3} M; bicarbonate ions, 5×10^{-3} M; L-glutamate, 3.8×10^{-3} M; no ammonia or bicarbonate ions were used with formamide, succinamate, and lactamide. The concentration of all these compounds was 4×10^{-3} M. Asparagine and leucine were tested in absence of ammonia and their concentrations were 1×10^{-3} M. α -Carbamylornithine, α -ketoglutaric semicarbazide, and α -ketoglutaric oxime were tested in presence of fumarate. Their concentrations were 1×10^{-3} M. Tissue concentrations, 2.24 mg. of N per flask. Incubation for 10 minutes at 38°. Final volume, 4.0 ml. Gas phase, air.

active, since glutamine and glutamic acid were equally active (Table I). In the absence of ammonia, carbamylglutamine is not at all active, indicating that the γ -amide group of this compound is not readily hydrolyzed. This finding is consistent with the observation by Krebs (29) that substitution in the α -amino group of glutamine prevents hydrolysis of the amide group by glutaminase. The γ -ethyl ester of carbamylglutamic acid showed the same order of activity as carbamylglutamine. L- α -Guanidinoglutaric acid proved to be inactive as far as citrulline synthesis is concerned and further failed to form arginine or urea in the system. The failure of α -carbamyl-DL-ornithine to form citrulline indicates that an

intramolecular transcarbamylation does not occur. The report by Lazarev (30) that carbamylglycine (hydantoic acid) is essential for citrulline synthesis is not supported by our findings with this compound. The analytical methods employed by Lazarev leave considerable doubt as to the

TABLE III
Effect of Carbamyl and Related Compounds on Synthesis of Citrulline

Relative rates expressed as per cent	
A	
L-Glutamate.....	100
Carbamyl-L-glutamate.....	260
“.....	
AMP replacing ATP.....	260
B	
Carbamyl-L-glutamate.....	100
Carbamyl-L-glutamic ester.....	35.0
Carbamyl-L-glutamine.....	32.5
Carbamyl-L-leucine.....	6
Formyl-L-glutamate.....	5
Phenylcarbamyl-L-glutamate.....	4
α-Carbamyl-DL-ornithine.....	3
5-Propionic hydantoin.....	2
Carbamylglycine.....	2
Carbamyl-DL-aspartate.....	0
Carbamyl-D-glutamate.....	0
Carbamyl-L-alanine.....	0
L-α-Guanidinoglutarate*.....	0

Final substrate concentrations, (A) magnesium sulfate, 6×10^{-3} M; potassium phosphate at pH 7.15, 1.3×10^{-2} M; fumarate or pyruvate, 3×10^{-2} M; glutamate or carbamyl-L-glutamate, 1×10^{-2} M; DL-ornithine, 2.5×10^{-3} M; ATP or AMP, 1.2×10^{-3} M; ammonium chloride, 4×10^{-3} M; bicarbonate ions, 5×10^{-3} M, and potassium ions to bring the medium to isotonicity. (B) same as for (A) except that carbamyl-L-glutamate or related compounds were at the concentration of 3.8×10^{-3} M; bicarbonate ions excluded. Tissue concentration, 2.9 mg. of N per flask. Final volume, 4.0 ml. Gas phase, air. (A) incubated for 20 minutes prior to the addition of ornithine. Incubation of the complete system, 10 minutes. (B) 35 minutes incubation of complete system. No preliminary incubation. System freed of CO₂.
* In absence of ammonia.

significance of his claims for the rôle of this compound. None of the carbamyl or the hydantoin compounds studied formed urea under the experimental conditions reported in the present study.
Threonine, leucine, valine, lysine, isoleucine, serine, glycine, methionine, alanine, glutamine, asparagine, and aspartic acid were used in the presence

of carbamyl-L-glutamate and compared with the value obtained with the latter compound alone. None of them has an appreciable effect upon the system.

The reported data show that carbamyl-L-glutamic acid is much more active than L-glutamic acid in the synthesis of citrulline and that compounds of a close structural relationship to carbamyl-L-glutamic acid have little or no activity. The high activity of carbamylglutamic acid has recently been confirmed by Leuthardt *et al.* (31) who, using a mitochondrial preparation from rat liver, found that the compound was 4 to 5 times more active than glutamic acid.

The comparative study of L-glutamic and carbamyl-L-glutamic acids in absence of bicarbonate ions previously reported (7) further indicates the highly specific rôle of the carbamyl compound, which was shown to be 15 to 20 times more effective than L-glutamic acid. The previously reported data (7) also show that carbamyl-L-glutamic acid is the limiting factor for citrulline synthesis in a wide range of concentrations. When the concentration of carbamyl-L-glutamic acid is raised, no increase in synthesis will occur after reaching the value for which the enzyme concentration is the limiting factor and a plateau is obtained. An increase in citrulline synthesis was observed with both an increase in enzyme concentration and in incubation time. If ornithine is omitted from these systems, there is no measurable formation of citrulline or urea and ammonia remains constant under the experimental conditions described.

The study with limiting ornithine concentrations shows essentially the same behavior. Work done on the effect of ammonia on the synthesis of citrulline from carbamyl-L-glutamic acid indicates that this substance does not act catalytically (7). The need for ammonia can again be seen clearly in Fig. 5 which shows that the synthesis of citrulline can occur from carbamyl-L-glutamic acid under anaerobic conditions, provided there is a preliminary incubation with oxygen and ammonia, prior to the addition of ornithine. The exclusion of ammonia in the preliminary aerobic incubation results in a striking decrease in the synthesis.

Properties of Enzyme System—Some properties relating to the stability of the enzyme system concerned with citrulline synthesis are listed in Table IV.

The washed residue from rat liver homogenate is relatively stable. No appreciable decrease in activity can be observed under the experimental conditions of the present study if the enzyme is incubated alone for a period of 20 to 30 minutes prior to the addition of substrates. If the incubation is continued, invariably after approximately 30 minutes there is a definite drop in activity. While the enzyme is unstable to freezing, it remains practically without loss in activity for 16 hours at 1–2°. Of in-

TABLE IV
Some Properties of Enzyme System

Relative rate expressed as per cent		
Glutamate	Carbamyl-L-glutamate	
<i>A</i>		
33.7	100.0	Aging enzyme at 38°, zero time
40.0	101.0	" " " 38°, 5 min.
33.0	100.0	" " " 38°, 10 "
32.3	100.0	" " " 38°, 20 "
31.2	96.0	" " " 38°, 25 "
29.1	84.0	" " " 38°, 29 "
19.1	60.0	" " " 38°, 32 "
<i>B</i>		
40.0	100.0	Storage at 1-2°, zero time
38.6	106.0	" " 1-2°, 1 hr.
37.2	112.0	" " 1-2°, 2 hrs.
34.3	104.0	" " 1-2°, 4 "
31.5	84.0	" " 1-2°, 8 "
34.9	92.0	" " 1-2°, 16 "
<i>C</i>		
36.0	100.0	Dialysis at 1-2°, zero time
14.0	62.2	" " 1-2°, 2 hrs.
13.0	52.2	" " 1-2°, 4 "
9.0	35.5	" " 1-2°, 8 "
0.15	0.55	" " 1-2°, 16 "
<i>D</i>		
26.6	70	Washed 1 time
32.1	100	" 2 times
32.3	97.5	" 3 "
27.0	85.0	" 5 "
23.6	75.0	" 7 "
20.1	65.0	" 9 "

Final substrate concentrations, magnesium sulfate, 6×10^{-3} M; ATP, 1×10^{-3} M; phosphate buffer pH 7.15, 1×10^{-2} M; DL-ornithine, 4×10^{-3} M; potassium fumarate, 8×10^{-3} M; ammonium ions, 2×10^{-3} M; glutamate or carbamyl-L-glutamate, 3.75×10^{-3} M; potassium ions to bring the medium to isotonicity. Incubation time 50 minutes at 38° in air. Final volume, 4.0 ml. Carbon dioxide was not excluded. Tissue concentrations, 4.5 mg. of N per flask. Citrulline synthesis under these conditions remains constant at 1.10 to 1.15 μ M citrulline per mg. of N when carbamyl-L-glutamate is used. These values, 1.10 to 1.15 μ M per mg. of N, are represented as 100. In the experiments of (A) the enzyme was kept in the side arm of Warburg vessels and incubated at 38° for the periods of time shown in the table before being mixed with the substrates. In (B) the enzyme was kept in the cold room for the period of time indicated in the table before incubation. (C), effect of dialysis on the enzyme system. (D), effect of repeated washings with isotonic KCl solution.

terest is the fact that a small increase in activity occurs during the first few hours of standing. A similar observation has been made previously by Cohen and McGilvery (32). Dialysis against isotonic KCl-phosphate of pH 7.15 at 1–2° leads to a relatively rapid loss in activity. On the other hand, the enzyme system appears to withstand up to nine washings with isotonic KCl with only a slight decrease in activity.

The reaction under study is not affected by concentrations of malonate in all ranges tested (up to 0.01 M) under the experimental conditions described in Table IV. This finding is in contrast to that observed with homogenates which are inhibited by malonate (5, 31).

It should be noted that parallel effects are observed with the procedures shown in Table IV with carbamyl glutamate and glutamate as substrates. This indicates that no loss has occurred in one or more of the enzymatic components effecting the conversion of glutamate to carbamyl glutamate.

Supplementation with TPN, DPN, biotin, or cytochrome *c* does not affect the synthesis of citrulline.

We have been unable to demonstrate the presence of an enzymatic system for the synthesis of citrulline, or arginine, in chicken and pigeon liver homogenates similar to the system present in mammalian liver.

DISCUSSION

Although the exact nature of the postulated intermediate (6, 7) in the reaction ornithine \rightarrow citrulline has not been unequivocally established, the data presented indicate that carbamyl-L-glutamic acid or a closely related compound is the active intermediate. Two experimental findings at present stand in the way of accepting carbamyl-L-glutamic acid as the intermediate. In the first place, the need for aerobic incubation for the synthesis of citrulline from carbamyl-L-glutamic acid plus ornithine in addition to the need for ATP is not consistent with the concept of a direct transcarbamylation reaction. However, it is possible that for some as yet unknown reason the transcarbamylation reaction requires ATP plus aerobic conditions. The second factor is that of the need for ammonia. If carbamyl-L-glutamic acid were the true intermediate, no ammonia should be required in the transcarbamylation reaction. However, the need for ammonia could be explained if the intermediate were a compound such as carbamylglutamine or guanidinoglutaric acid, or if the carbamyl-glutamic acid were rapidly hydrolyzed to form carbon dioxide and ammonia and the latter utilized by competing systems. In regard to the first possibility, both carbamylglutamine and guanidinoglutaric acid are inactive in the absence of ammonia. Regarding the second possibility, it would be expected that extra ammonia would be required so as to insure rapid resynthesis of carbamylglutamic acid. Experiments bearing

on this possibility revealed that carbamylglutamic acid did not form free ammonia when incubated with washed residue. Thus the basis for the need for ammonia remains unanswered. Of interest is the observation from balance studies that for each mole of citrulline synthesized from glutamic acid plus ornithine 2 moles of ammonia are taken up, while citrulline synthesis from carbamylglutamic acid plus ornithine results in the uptake of only 1 mole of ammonia under the experimental conditions shown in Table IV. It is worthy of note that the extra ammonia used in the system forms a stable compound that is not hydrolyzed by acid at room temperature for 24 to 48 hours or by alkali at 60° for 10 minutes. Further, determination of carbamylglutamic acid disappearance in the same system indicated a decrease of this compound coincident with citrulline synthesis. From these observations it must be concluded that the carbamyl group as such is utilized in the synthesis of citrulline. The study of isotopically labeled compounds should provide more direct evidence for this reaction. Such studies are now in progress.³

SUMMARY

1. The intermediate steps in the conversion of ornithine to citrulline include the formation of carbamylglutamic acid, or a closely related compound, from glutamic acid, carbon dioxide, and ammonia. This endergonic reaction requires adenosine triphosphate. A transcarbamylation reaction then occurs as a result of which the carbamyl group is transferred to the δ -amino group of ornithine. Under suitable conditions the transcarbamylation reaction can be shown to take place anaerobically.

2. Carbamylglutamic acid, while more active than glutamic acid under all conditions in citrulline synthesis, is not active in the absence of ammonia. Carbamylglutamine and guanidinoglutaric acid are also inactive in the absence of ammonia. Of a series of carbamyl derivatives of amino acids studied, carbamylglutamic acid alone is highly active.

3. Some properties relating to the stability of the enzyme system are reported.

BIBLIOGRAPHY

1. Krebs, H. A., *Ann. Rev. Biochem.*, **5**, 262 (1936).
2. Cohen, P. P., and Hayano, M., *J. Biol. Chem.*, **172**, 405 (1948).

³ Since this paper has been submitted for publication, a soluble enzyme has been prepared from acetone-dried washed residue followed by low temperature alcohol fractionation. This enzyme, termed transcarbamylase, catalyzes the synthesis of citrulline *anaerobically* from carbamylglutamic acid but not appreciably from glutamic acid plus CO₂ and ammonia. The details of this reaction system will be published in the near future.

3. Leuthardt, F., and Brunner, R., *Helv. chim. acta*, **30**, 958 (1947).
4. Leuthardt, F., and Glasson, B., *Helv. chim. acta*, **25**, (1942); *Helv. physiol. Acta*, **2**, 549 (1944).
5. Cohen, P. P., and Hayano, M., *J. Biol. Chem.*, **170**, 687 (1947).
6. Cohen, P. P., and Grisolia, S., *Federation Proc.*, **7**, 150 (1948).
7. Cohen, P. P., and Grisolia, S., *J. Biol. Chem.*, **174**, 389 (1948).
8. LePage, G. A., in Umbreit, W. W., Burris, R. H., and Stauffer, G. F., *Manometric techniques and related methods for the study of tissue metabolism*, Minneapolis (1945).
9. Kerr, S. E., *J. Biol. Chem.*, **139**, 131 (1941).
10. Williamson, S., and Green, D. E., *J. Biol. Chem.*, **135**, 345 (1940).
11. Warburg, O., Christian, W., and Griese, A., *Biochem. Z.*, **282**, 157 (1935).
12. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **122**, 298 (1937).
13. Wisclienus, W., and Waldmuller, M., *Ber. chem. Ges.* **44**, 1564 (1911).
14. Neuberg, C., and Ringer, M., *Biochem. Z.*, **71**, 228 (1915).
15. Simon, L. S., *Compt. rend. Acad.*, **137**, 855 (1903).
16. Lardy, H. A., in Umbreit, W. W., Burris, R. H., and Stauffer, G. F., *Manometric techniques and related methods for the study of tissue metabolism*, Minneapolis (1945).
17. Nyc, G. F., and Mitchell, H. K., *J. Am. Chem. Soc.*, **69**, 1382 (1947).
18. Hopkins, S. J., and Wormall, A., *Biochem. J.*, **28**, 228 (1934).
19. Carter, H. E., and West, H. D., *Org. Syntheses*, **20**, 101 (1940).
20. Bergmann, M., and Zervas, L., *Z. physiol. Chem.*, **221**, 51 (1933).
21. Wheeler, H. L., and Merriam, H. F., *Am. Chem. J.*, **29**, 478 (1903).
22. Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, **210**, 33 (1932).
23. Archibald, R. M., *J. Biol. Chem.*, **157**, 507 (1945).
24. Archibald, R. M., *J. Biol. Chem.*, **156**, 121 (1944).
25. Hunter, F. E., *Federation Proc.*, **8**, 208 (1949).
26. Krebs, H. A., Eggleston, L. V., and Heins, R., *Biochem. J.*, **43**, 406 (1948).
27. Weil-Malherbe, H., and Krebs, H. A., *Biochem. J.*, **29**, 2077 (1935).
28. Taggart, J. V., and Krakaur, R. B., *J. Biol. Chem.*, **177**, 641 (1949).
29. Krebs, H. A., *Biochem. J.*, **29**, 1951 (1935).
30. Lazarev, A. F., *Doklady Akad. Nauk S. S. S. R.*, **61**, 677 (1948).
31. Leuthardt, F., Müller, A. F., and Nielsen, H., *Helv. chim. acta*, **32**, 744 (1949).
32. Cohen, P. P., and McGilvery, R. W., *J. Biol. Chem.*, **171**, 121 (1947).

NUCLEOTIDE PYROPHOSPHATASE

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The wide-spread occurrence of enzymes which split diphosphopyridine nucleotide (DPN) has been appreciated for many years (1), but investigation of the mechanisms of cleavage has been undertaken only recently. In rabbit tissues, two distinct pathways for the breakdown of DPN exist (2). DPN nucleosidase, described by Handler and Klein (3), catalyzes the cleavage of the glycosidic bond between nicotinamide and ribose. DPN pyrophosphatase (2), on the other hand, catalyzes the cleavage of the pyrophosphate bond between adenylic acid and nicotinamide mononucleotide (NMN). Nicotinamide specifically inhibits the nucleosidase and provides a simple way of distinguishing between these two modes of DPN breakdown.

An enzyme has been purified from potato extracts that splits the pyrophosphate linkage of DPN and also those of flavin-adenine dinucleotide (FAD), triphosphopyridine nucleotide (TPN), and adenosine triphosphate (ATP) (4). This enzyme has made NMN and riboflavin phosphate readily available for use in studies of the biosynthesis of DPN (5, 6) and FAD (7) and has provided new information concerning the structure of TPN (4).

In this report a detailed study of the purification and properties of nucleotide pyrophosphatase is presented.

Methods

Materials—DPN, reduced DPN (DPNH_2), TPN, ATP, adenosine diphosphate (ADP), FAD,¹ adenosine-5-phosphate, and glycylglycine are described in subsequent papers (6, 7). NMN was prepared as described below. Adenosine-3-phosphate was a Schwarz product, glycerophosphate (α , 52 per cent) an Eastman Kodak product, and thiamine pyrophosphate (cocarboxylase) a Merck sample. Glucose-6-phosphate (8)² and *dl*-isocitric acid (9)³ were synthetic materials.

Crystalline alcohol dehydrogenase was prepared from bakers' yeast according to Racker,⁴ lactic dehydrogenase was purified from rabbit muscle,

¹ Kindly furnished by Dr. L. A. Heppel.

² Kindly furnished by Dr. B. L. Horecker.

³ Kindly furnished by Dr. Severo Ochoa.

⁴ Personal communication from Dr. E. Racker.

and isocitric dehydrogenase was a dialyzed phosphate extract of heart muscle acetone powder prepared according to Ochoa (10).

Determinations—DPN and TPN were determined with the model DU Beckman spectrophotometer by measuring the increase in optical density at 340 $m\mu$ after enzymatic reduction. DPN was reduced (6) with alcohol and crystalline alcohol dehydrogenase and TPN with *dl*-isocitric acid and isocitric dehydrogenase (10).

Other determinations were as follows: $DPNH_2$ by oxidation with pyruvic acid and lactic dehydrogenase (11); inorganic phosphate according to Lowry and Lopez (12); total phosphate, which included the phosphate liberated after ashing with an H_2SO_4 - HNO_3 mixture, by the method of Fiske and Subbarow (13); adenosine-5-phosphate by Kalckar's method (14); FAD and riboflavin phosphate fluorometrically (15); nicotinamide-ribose (N-R) moiety fluorometrically (16); and protein by the biuret procedure (17) for crude preparations and by ultraviolet absorption (18) for purified preparations.

Assays of Nucleotide Pyrophosphatase Activity—A unit of enzyme activity is defined (except in the case of FAD splitting) as the amount causing the splitting of 1 μM (micromole) of substrate per hour and specific activity as units per mg. of protein. Assays were at 38°. In tests of TPN-, FAD-, and ATP-splitting activity, the substrate concentrations used were suboptimal and the rates obtained were therefore submaximal.

DPN-Splitting Activity—Enzyme purification was carried out on the basis of this activity. An incubation mixture of 1.0 cc. contained 0.1 cc. of DPN (0.02 M, pH 6), 0.2 cc. of potassium phosphate buffer (0.5 M, pH 7.0), and from 1 to 5 enzyme units. After incubation for 20 minutes, 0.1 cc. of incubation mixture was added to each of two absorption cells (1 cm.) containing 2.8 cc. of an alcohol-pyrophosphate buffer mixture (6). After an initial reading, 0.1 cc. of water was added to one cell ("blank") and 0.1 cc. of alcohol dehydrogenase (5 to 10 γ) to the other. Complete reduction was reached after about 5 minutes. Solutions of DPN, adjusted to pH 6, were stable for weeks at 0°.

$DPNH_2$ -Splitting Activity—The incubation mixture of 0.25 cc. contained 0.05 cc. of $DPNH_2$ (0.01 M, pH 7.4), 0.05 cc. of potassium phosphate buffer (0.5 M, pH 7.0), and 1 to 4 enzyme units. After 20 minutes, 0.1 cc. of incubation mixture was added to each of two absorption cells (1 cm.) containing 2.9 cc. of pyruvate-phosphate buffer mixture (11). After an initial reading, 0.01 cc. (2 to 5 γ) of lactic dehydrogenase was added to one cell ("blank") and 0.01 cc. of water to the other. Complete oxidation with stable readings was attained within 30 seconds.

TPN-Splitting Activity—The incubation mixture of 0.2 cc. contained 0.05 cc. of TPN (0.004 M, pH 6), 0.04 cc. of glycylglycine buffer (0.25 M,

pH 7.4), and 1 to 4 enzyme units. After 20 minutes incubation in an absorption cell, 2.75 cc. of isocitrate-glycylglycine mixture (10) were added. An initial reading was taken against a blank cell containing water in place of the incubation mixture. Then 0.05 cc. (0.12 mg.) of isocitric dehydrogenase was added to both cells. Complete reduction with stable readings was attained after 2 to 3 minutes.

FAD-Splitting Activity—The incubation mixture of 10.0 cc. contained 0.05 cc. of FAD (1.8 γ of an 85 per cent pure barium salt), 9.0 cc. of potassium phosphate buffer (0.05 M, pH 7.4), and 3 to 10 units of enzyme. The incubation was at room temperature (22–25°). Readings were taken for 3 minutes at 30 second intervals with a model 12-A Coleman photofluorometer. A blank tube with no enzyme added was read at similar intervals and provided a check on the stability of the instrument. A unit of enzyme activity is defined as the amount causing an increase of 1 galvanometer unit per minute. The sensitivity of the instrument based on an FAD standard did not vary significantly during the course of this investigation.

ATP-, ADP-, and Thiamine Pyrophosphate-Splitting and Other Phosphatase Activities—The incubation mixture of 1.0 cc. contained 0.2 cc. of 0.02 M neutralized substrate, 0.2 cc. of glycylglycine buffer (0.25 M, pH 7.4), and an amount of enzyme calculated to release approximately 0.5 μ M of phosphate in 10 minutes. The reaction was stopped after 10 minutes by addition of 1.0 cc. of 5 per cent trichloroacetic acid. Turbidity was removed by centrifugation and the supernatant analyzed for its inorganic phosphate content. The phosphate release from ATP was considered to be a close approximation to the ATP removal, since the ATP- and ADP-splitting activities of all enzyme fractions were similar and since only 10 to 15 per cent of the ATP was removed in the test.

Results

Purification of Enzyme

Purification Procedure—This included the following steps: (1) extraction, (2) ammonium sulfate fractionation, (3) ethanol fractionation, and (4) adsorption on calcium phosphate gel and elution. DPN-splitting activity was the basis for purification.

Extraction—200 gm. of peeled Maine potatoes were extracted with 400 cc. of 0.40 saturated ammonium sulfate for 90 seconds in a Waring blender. The extract was filtered on fluted papers at 2°. From 5 kilos of potatoes, 10 liters of filtrate were obtained. The use of Celite as a filter aid resulted in a loss of enzyme activity. The yield obtained by extraction of potatoes with 2 volumes of water (aqueous extract, Table I) approximated that of the ammonium sulfate fraction.

Ammonium Sulfate Fractionation—To 10 liters of filtrate were added 2

kilos of solid ammonium sulfate. Filtration was at 2° through a 50 cm. fluted paper with an arrangement for automatic refilling to permit collection of the precipitate on a single paper and completion of the filtration overnight. The brownish black precipitate was scraped from the paper and dissolved with water to a volume of 550 cc. The dark solution was dialyzed against running tap water (at 8–18°) for 90 minutes in cellophane

TABLE I
Purification of Nucleotide Pyrophosphatase

Step	Ethanol (95 per cent) added	Volume of fraction	Total activity	Yield	Specific activity
	cc.	cc.	units	per cent	units per mg. protein
Aqueous extract			350,000		2.9
Ammonium sulfate.....		2750	317,000	91	5.4
Ethanol Fraction I-1.....	397	1000	58,500	18	1.8
“ “ I-2.....	438	680	230,000	72	15.5
“ “ II-2a.....	41	178	15,850	7	2.0
“ “ II-2b.....	27	160	61,000	27	15.6
“ “ II-2c.....	30	162	125,000	54	84.7
“ “ III-2c-1.....	44	36	13,300	11	18.8
“ “ III-2c-2.....	11	36	34,200	27	76.5
“ “ III-2c-3.....	5.5	30	30,700	24	202
“ “ III-2c-4*.....		25	21,400	17	278
“ “ III-2c-5†.....		21	25,700	20	216
Calcium phosphate, 1st adsorption					
Fractions 2c-3, 4, 5.....		254	77,500		220
Eluate 1.....		100	52,000	67	1625
“ 2.....		100	7,700	10	1065
“ 3.....		100	910	1	530
Calcium phosphate, 2nd adsorption, eluate.....		75	38,300	74	2200

* Fraction 2c-4 was the precipitate which appeared in the supernatant of Fraction 2c-3 after standing at –10° for 3 hours.
† Fraction 2c-5 was the precipitate which appeared in the supernatant of Fraction 2c-4 after standing at –10° for 18 hours.

sacs. The volume after dialysis was 660 cc. and the pH 5.5 to 5.6. Four such batches of dialyzed ammonium sulfate fractions were combined (ammonium sulfate, Table I) and fractionated with ethanol.
Ethanol Fractionation—The dialyzed ammonium sulfate fraction was brought to pH 4.4 with acetic acid (61 cc. of 1 M). The solution was cooled to –0.5° and 95 per cent ethanol was added with mechanical stirring. The temperature was maintained just above the freezing point

during the early ethanol additions and at -5° thereafter. The precipitates were centrifuged off at 0° and dissolved in water. Fraction 2 was refractionated as indicated (Table I).

These two fractionations have been carried out four times with little variation from the results of the first trial. Attempts to standardize a third ethanol fractionation were unsuccessful. Fractions with high specific activity were obtained in good yield, but minor variations in temperature, time, and speed of ethanol addition influenced the amount of ethanol required. Accordingly, this fractionation was carried out by collecting several ethanol fractions and combining the best (Table I).

Calcium Phosphate Adsorption—The combined ethanol fractions (Nos. 2c-3, 4, and 5) (pH 4.4) were diluted with water to give a protein concentration of 1.5 mg. per cc. Calcium phosphate gel (19) (202 cc., aged 2 months, dry weight 7.9 mg. per cc.) was added and the mixture was stirred mechanically for 5 minutes at room temperature. The precipitate was collected by centrifugation and washed 4 times with 100 cc. of 0.1 M potassium phosphate buffer, pH 7.4. The enzyme was eluted with three portions of 100 cc. of 0.20 saturated ammonium sulfate adjusted with ammonia water to pH 7.5.

To concentrate Eluates 1 and 2 to a small volume, 36 gm. of solid ammonium sulfate were added to each. The precipitates, collected in a high speed centrifuge, were dissolved in water to yield a protein concentration of 2 mg. per cc. The yield in this step was 92 per cent and the specific activity was unaltered.

A second calcium phosphate adsorption increased the specific activity to 2200 units per mg. with a yield of 80 per cent. 15 cc. of the ammonium sulfate concentrate of Eluate 1 above were diluted to 300 cc., adsorbed with 15 cc. of calcium phosphate gel, washed 3 times with 150 cc. of 0.05 M potassium phosphate buffer, pH 7.0, and eluted with 75 cc. of 0.20 saturated ammonium sulfate, pH 7.5.

The entire purification procedure resulted in a 750-fold purification with an over-all yield of 11 per cent. The yield may be improved by combining and reprocessing some fractions of lower purity. The term "purified enzyme" in this report refers to a calcium phosphate eluate (or ammonium sulfate concentrate) with a specific activity of 1625 units or more per mg. and "crude enzyme" to the aqueous extract (Table I).

Variations in Activity in Potatoes—The age and variety of potato influenced the nucleotide pyrophosphatase activity (Table II). Eleven varieties of Maine potatoes, which were harvested at the same time and stored under identical conditions prior to the initial assay, differed widely (3.3 to 21.9 units per cc.). Aging at 3° resulted in a variable increase in activity. Since the protein concentration was relatively uninfluenced by

the variety of potato or its age, the specific activity (units per mg. of protein) reflected the enzyme activity. The low activity was not due to the presence of an inhibitor, since an extract of a potato of high activity (55.0 units per cc.) tested in the presence of an equal volume of an extract of a potato of low activity (3.3 units per cc.) was not inhibited.

TABLE II

Nucleotide Pyrophosphatase in Several Varieties of Maine Potatoes

The potatoes were from the United States Department of Agriculture farm in Maine and obtained through the courtesy of Dr. R. Stevenson in Beltsville, Maryland, in October, 1948. They were stored at 3° after initial assay. For assay, two or more potatoes of each variety were taken at random, peeled, and diced, and a 100 gm. aliquot homogenized with 200 cc. of 0.40 saturated ammonium sulfate. To 30 cc. of filtrate were added 6 gm. of ammonium sulfate and the precipitate, collected by centrifugation, was dissolved in water to a volume of 5.0 cc. DPN-splitting activity was determined.

Variety	Content, units per cc.			Specific activity, units per mg. protein		
	0 wk.	6 wks.	16 wks.	0 wk.	6 wks.	16 wks.
Chippewa.....	3.3	9.0	5.5	0.4	1.1	0.6
Triumph.....	5.9	6.9	7.3	0.7	0.6	0.6
Irish Cobbler.....	9.9	12.9	17.0	1.1	1.4	1.5
Houma.....	12.5	21.2	20.8	1.4	2.6	1.6
Green Mountain.....	13.4	18.0	17.0	2.3	2.6	2.2
Sebago.....	14.0	26.4	25.2	1.1	2.2	3.6
White Rose.....	14.6	24.9	41.7	1.6	4.0	4.0
Teton.....	16.5	34.6	40.1	2.3	3.5	3.8
“ *.....		21.6	24.2		2.3	2.5
Katahdin.....	20.7	26.0	25.6	1.6	2.7	2.3
Rural New Yorker.....	21.0	23.1	29.4	2.5	5.9	3.2
Russet Burbank.....	21.9	27.0	18.2	3.1	4.7	2.1
Average.....	14.0	21.0	22.7	1.6	2.8	2.3
“ protein concentration, mg. per cc.....	8.7	7.5	9.9			

* Obtained from a later harvest.

Stability—The enzyme is remarkably stable at 0–5°. Preparations of varying purity have shown no detectable loss of activity over a period of 15 months. However, some dilute solutions (50 γ of protein per cc.) lost 50 per cent of their activity in 5 days. There was no inactivation on incubation for 20 minutes at 38° at pH 3.2 in 0.1 M citrate buffer or at pH 9.3 in 0.1 M glycine buffer. There was complete inactivation on incubation for 15 minutes at 38° at pH 12.5, or for 10 minutes at 38° at pH

1.4. The enzyme was not inactivated by freezing or by a 9 hour dialysis against running distilled water.

DPN Splitting—The reaction involves hydrolysis of the pyrophosphate bond to yield NMN and adenosine-5-phosphate as indicated in Table III. The removal of DPN was accompanied by the appearance of a nearly equivalent amount of adenosine-5-phosphate. There was no change in the orthophosphate concentration and no decrease of the nicotinamide-ribose (N-R) moiety. Indeed, a slight increase in the N-R value, which has been noted previously in DPN splitting by kidney (2), may indicate a higher specific fluorescence coefficient for NMN as compared with DPN. As indicated below, the hydrolysis of DPN goes to completion.

A large scale preparation of NMN was carried out as follows: To a solution of 500 mg. of DPN adjusted to pH 6 were added 0.4 cc. of 0.5 M

TABLE III
Balance Study of DPN Splitting

The experimental incubation mixture (1.0 cc.) contained 1.93 μM of DPN, 50 μM of glycylglycine buffer, pH 7.4, and 4 γ of purified enzyme. After 15 minutes at 38°, the enzyme was inactivated by incubating with 0.1 cc. of 1 N HCl for 10 minutes at 38°. The reaction mixture was then neutralized with 0.05 cc. of 2 N NaOH. The values are expressed in micromoles. The enzyme was free of the substances assayed below.

	DPN	Adenosine-5-phosphate	Ortho-phosphate	Nicotinamide-ribose moiety
Control (no enzyme).....	1.93	0.05	0.08	1.93
Experimental.....	0.28	1.56	0.08	2.31
Δ	-1.65	+1.51	0.00	+0.38

potassium phosphate buffer (pH 7.7) and 1.0 mg. of purified nucleotide pyrophosphatase in a final volume of 5.5 cc. After a 90 minute incubation at 38°, no detectable DPN remained ($<0.05 \mu\text{M}$). Basic lead acetate (1 M) was added in slight excess (1.0 cc.), the precipitate discarded, and the pH of the supernatant fluid adjusted to 3.0 with dilute nitric acid. A small precipitate was removed after addition of 0.5 cc. of 20 per cent mercuric acetate. The lead and mercury were removed from the supernatant fluid with H_2S . The clear, colorless solution contained no DPN, inorganic orthophosphate, or adenine nucleotides. The ratio of N-R moiety, estimated fluorometrically (16), to phosphate was 0.9. The yield of NMN was approximately 80 per cent.

The dissociation constant (K_s) (20) of the DPN-enzyme complex, calculated from the data in Fig. 1, a is 1.5×10^{-4} mole per liter. The pH dependence of the reaction is shown in Fig. 2. There was a broad opti-

imum between pH 6.5 and 8.5 and a decrease of activity of about 50 per cent at pH 4.0 and 9.0. Enzyme inactivation did not contribute to this curve, since full activity was obtained on neutralization of enzyme that

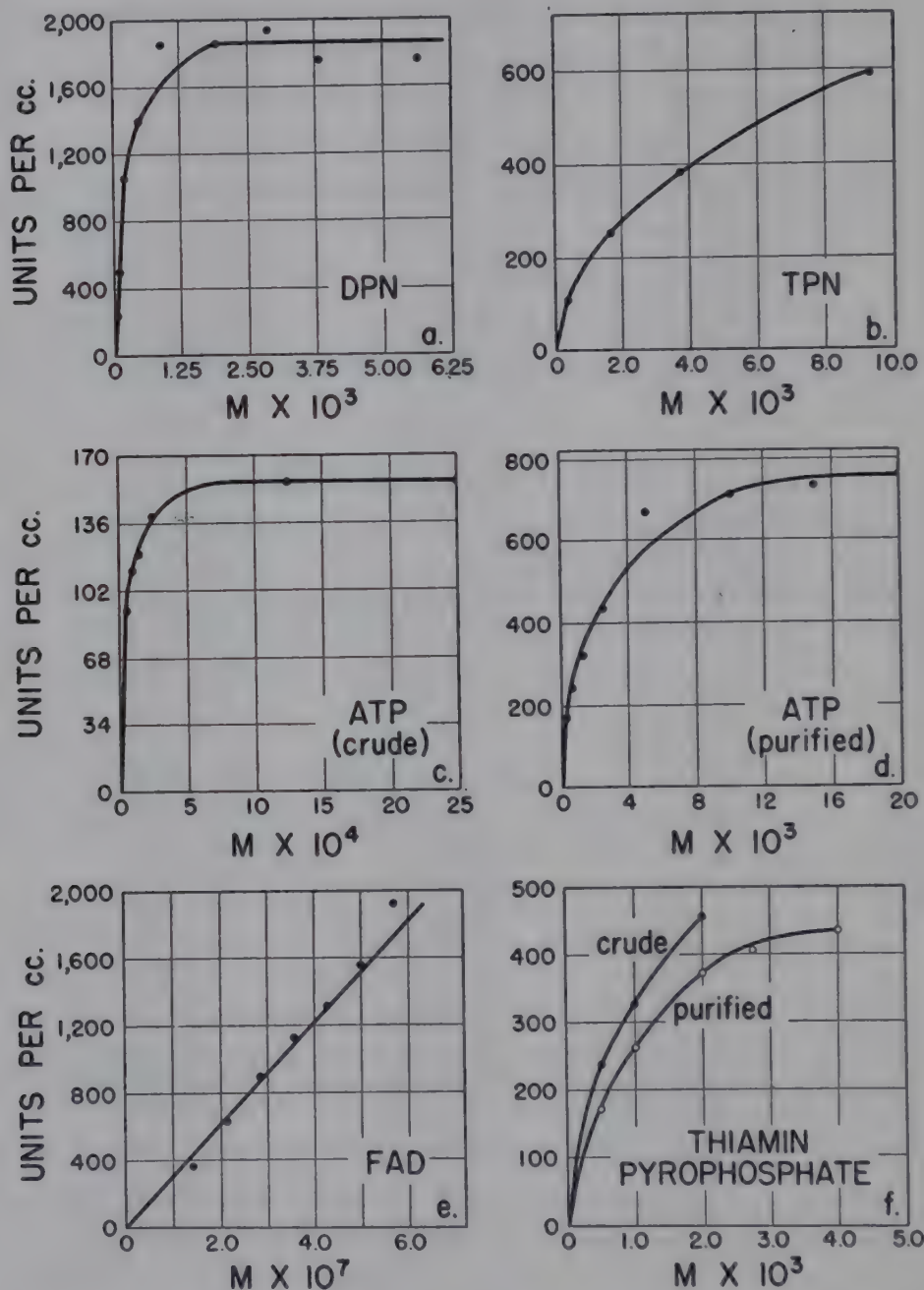


FIG. 1. Substrate affinities for nucleotide pyrophosphatase. Purified enzyme (1 mg. per cc.) was used except when crude aqueous extract is specified. The amount of enzyme used was so adjusted that the extent of splitting at suboptimal substrate concentrations did not exceed 20 per cent of the substrate.

had been incubated for 20 minutes at 38° at pH 3.2 or 9.3. Neither was the pH curve a reflection of the varying concentration of an active ionic species of DPN. The activities at pH 3.4 and 9.2 determined in the presence of a 2-fold increase (0.02 M to 0.04 M) in DPN concentration were

not significantly altered. Borate and pyrophosphate inhibited strongly at pH 8.5 but not at pH 7.4, suggesting that the quaternary pyrophosphate and tertiary borate ions were the active inhibiting agents.

Several other compounds were tested for a possible inhibitory effect on DPN splitting with the following results. In glycylglycine buffer (0.05 M, pH 7.4) at concentrations of 0.04 M, adenosine-3-phosphate, glucose-6-phosphate, and glycerophosphate had no effect; adenosine-5-phosphate decreased the rate by 49 per cent and NMN by 10 per cent. Nicotinamide (0.1 M) produced an inhibition of 19 per cent.

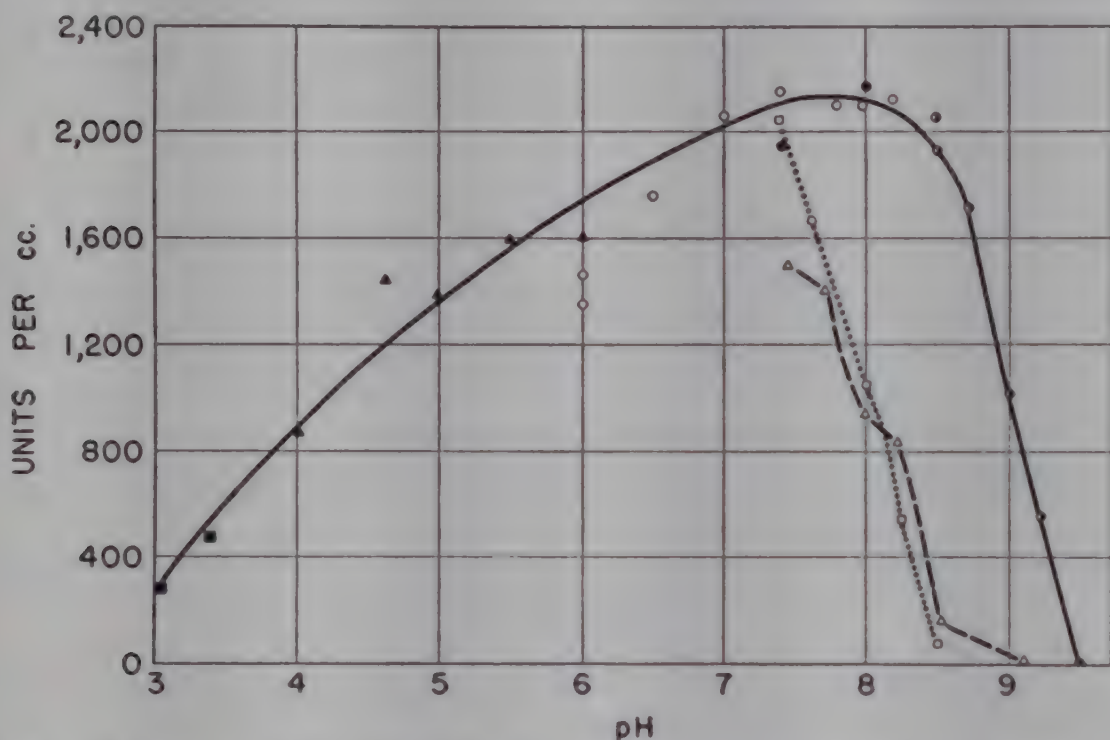


FIG. 2. Influence of pH on DPN splitting by nucleotide pyrophosphatase. The buffers used were 0.05 M and are designated as follows: citrate ■, acetate ▲, phosphate ○, glycylglycine ●, glycine ○, pyrophosphate □, borate △. Purified enzyme (1 mg. per cc.) was used.

DPNH₂ Splitting—This appears to be a hydrolysis of the pyrophosphate bond to yield the reduced mononucleotide (NMNH₂) and adenosine-5-phosphate (Table IV). NMNH₂ has the same absorption coefficient as DPNH₂ at 340 mμ, since no change in density was noted after the partial or complete cleavage of DPNH₂. Unlike DPNH₂, it is not oxidized by the pyruvate-lactic dehydrogenase system. NMNH₂ is also less stable than DPNH₂. After 1 week at pH 7.4 at 0°, the concentration of DPNH₂ decreased by 14 per cent, while that of NMNH₂ decreased by 49 per cent. With yeast autolysates and liver and kidney homogenates which rapidly oxidized DPNH₂, little or no oxidation of NMNH₂ was observed. The rate of DPNH₂ splitting was 647 units per mg. of protein, as compared

with 1320 units per mg. of protein for DPN splitting at a comparable substrate concentration.

TPN Splitting—In previous work (4), it was found that the splitting of TPN results in the production of NMN and an adenine nucleotide (not ADP) containing two phosphate groups. Evidence was thus provided that the TPN molecule contained only 1 pyrophosphate bond and that this was split by nucleotide pyrophosphatase.

Available data indicate that a single enzyme hydrolyzes both DPN and TPN. From Table V, it may be seen that the ratio of DPN-splitting to TPN-splitting activities was relatively constant in preparations differing widely in specific activity. It has not been possible to state a ratio for the crude aqueous extract, owing to the presence of an enzyme which converts TPN to DPN. Such a conversion has been described in yeast

TABLE IV
Balance Study of DPNH₂ Splitting

The experimental incubation mixture (0.25 cc.) contained 0.52 μM of DPNH₂, 25 μM of phosphate buffer, pH 7.0, and 4 γ of purified enzyme. After 10 minutes at 38°, the enzyme was inactivated by incubating with 0.04 cc. of 2 N NaOH for 10 minutes at 38°. The reaction mixture was then neutralized with 0.04 cc. of 2 N HCl. A 0.1 cc. aliquot was analyzed. The optical density is $\log I_0/I$; other values are expressed in micromoles. The enzyme was free of substances determined below.

	Optical density		DPNH ₂	Adenosine-5-phosphate
	Before lactic dehydrogenase	After lactic dehydrogenase		
Control (no enzyme).....	0.423	0.094	0.157	0.021
Experimental.....	0.418	0.364	0.026	0.143
Δ			-0.131	+0.122

(21). The accumulation of DPN from TPN breakdown with the aqueous extract was at the rate of 1.0 μM per cc. per hour; the rate of DPN splitting by this extract was 1.9 μM per cc. per hour. The slight increase in the ratio of DPN splitting to TPN splitting during the course of purification is very likely the result of the removal of this phosphatase activity.

The K_s for TPN and maximal rate of TPN splitting calculated from a Lineweaver-Burk plot (22) of the data in Fig. 1, *b* were 3.0×10^{-3} mole per liter and 805 units per cc. respectively. The maximal rate for DPN splitting by the same enzyme preparation was 1900 units per cc. As might be anticipated from their relative K_s values, DPN is an effective inhibitor of TPN splitting. With TPN and DPN present in equimolar concentrations, the rate of TPN splitting was reduced from 78 to 6 units per cc. and that of DPN splitting from 276 to 261 units per cc.

FAD Splitting—The splitting of FAD by potato extracts to yield the constituent mononucleotides was first observed by Lowry, Bessey, and Love.⁵ This observation has now been confirmed with the purified nucleotide pyrophosphatase. In an experiment in which $0.063\ \mu\text{M}$ of FAD was completely hydrolyzed as determined by the increase in fluorescence, $0.062\ \mu\text{M}$ of adenosine-5-phosphate appeared. The quantitative production of riboflavin phosphate from FAD splitting is indicated in a subsequent paper (7).

Evidence for the identity of the DPN- and FAD-splitting enzymes is given in Table V, where it is seen that the ratio of these two activities is remarkably constant during the course of extensive purification. Since the expression of FAD-splitting activity in terms of fluorometer readings

TABLE V

Various Pyrophosphatase Activities at Different Stages of Enzyme Purification

The values are ratios of the DPN-splitting activity to the activity in the splitting of the compounds indicated. The ratios are not a direct measure of the relative potency of nucleotide pyrophosphatase for these substrates (see the text).

Specific activity for DPN splitting, units per mg. protein	TPN	FAD	ATP	ADP	Thiamine pyrophosphate
3*		1.9	0.02	0.01	0.04
5†	2.3	2.9	0.05	0.06	0.20
16	2.8	2.1	0.18	0.22	0.51
80	3.7	1.8	0.79	0.70	1.4
162	5.0	1.6	2.2	2.8	3.7
1665	5.8	2.1	4.2	4.5	4.7
2200	5.0	1.5	3.7	4.6	4.7

* Aqueous potato extract.

† Ammonium sulfate fraction.

does not permit a comparison with the other nucleotide-splitting potencies, the following determinations were made. An incubation mixture of 0.20 cc. contained 0.05 cc. of FAD ($1.3 \times 10^{-4}\ \text{M}$), 0.04 cc. of glycylglycine buffer (0.25 M, pH 7.4), and from 0.06 to 0.12 γ of purified enzyme. After 10 minutes at 38° , the sample was diluted to 50 cc. with 0.05 M phosphate buffer, pH 7.4, and read in the fluorometer. From the fluorometer readings obtained simultaneously for comparable FAD solutions without added enzyme and with excess enzyme (0.5 γ), the extent of FAD splitting was readily determined. Thus at an FAD concentration of $3 \times 10^{-5}\ \text{M}$, the rate of splitting was $201\ \mu\text{M}$ per mg. of protein per hour. With the same enzyme preparation, the splitting of DPN was $87\ \mu\text{M}$ per mg. per hour at $4.6 \times 10^{-5}\ \text{M}$ and 1900 at $2.0 \times 10^{-3}\ \text{M}$ (Fig. 1, a).

⁵ Personal communication from Dr. O. H. Lowry.

At very low FAD concentrations (convenient for direct measurement), there was a linear relationship between the concentration of FAD and the rate of hydrolysis as measured by increase in fluorescence (Fig. 1, *e*). A reliable approximation of the K_s cannot be made from these data. Under routine assay conditions, DPN in a molar ratio of approximately 20 (5×10^{-5} M DPN) produced no significant inhibition of FAD splitting, but in a molar ratio of approximately 400 (1×10^{-3} M DPN) there was an inhibition of 76 per cent.

Studies with D-amino acid oxidase suggest that nucleotide pyrophosphatase does not split FAD bound as a prosthetic group. In the presence of an amount of pure D-amino acid oxidase (23)¹ calculated from its dissociation constant (24) to reduce the free FAD content by 46 per cent, the observed reduction in the rate of splitting was 32 per cent. In view of certain approximations involved in this calculation, this may be considered as fair agreement. With reduced FAD (reduced by incubation with alanine and the enzyme), which is considered to have a far lower dissociation constant than oxidized FAD (24), there was no detectable splitting action by nucleotide pyrophosphatase.

ATP and ADP Splitting—The presence in crude potato extracts of a potent adenylypyrophosphatase was first described by Kalckar (25) and has been studied more recently by Krishnan (26). During the course of purification of nucleotide pyrophosphatase, the activity toward ATP and ADP was rapidly reduced to a very low level, but reached a constant value which could not be further reduced (4) (Table V). The evidence below gives additional support to the view that hydrolysis of the pyrophosphate bonds of ATP (and very likely of ADP) is a property of nucleotide pyrophosphatase.

The K_s of the ATP-splitting enzyme was determined for the crude aqueous extract and for the purified enzyme and found to be 0.03×10^{-3} and 2.0×10^{-3} mole per liter, respectively (Fig. 1, *c* and *d*). These markedly different affinities establish the existence of two different ATP-splitting enzymes. It might be considered further that, if the ATP-splitting activity of the purified enzyme and nucleotide pyrophosphatase were identical, ATP should inhibit the hydrolysis of DPN and that its affinity for the enzyme as an inhibitor (K_i) (22) should be the same as its affinity as a substrate (K_s). From Table VI, it can be seen that the values for K_i (2.7 to 3.1×10^{-3} mole per liter) closely approximate the K_s value. Finally, it was shown that small amounts of DPN strongly inhibited ATP splitting by the purified enzyme, while much larger amounts had no effect on ATP splitting by the crude enzyme. DPN in a molar ratio of 0.16 (4×10^{-4} M) produced a 56 per cent inhibition of ATP splitting by the purified enzyme. (A K_i value for DPN was not determined because of

the rapid splitting of DPN.) DPN in a molar ratio of 1.6 (20×10^{-4} M) did not alter the rate of ATP splitting by the crude enzyme.

From Fig. 1, *d*, the calculated maximal rate of ATP splitting is approximately 760 units per cc. as compared with a maximal rate of 1900 units per cc. for DPN splitting, thus giving a ratio of 2.5. The higher ratios of Table V were due to the use of suboptimal levels of ATP in the routine assay.

Thiamine Pyrophosphate Splitting—As with adenylypyrophosphatase, there is an active thiamine pyrophosphatase in crude potato extracts. While the bulk of this activity is readily separated, a significant residue remains which is not significantly decreased by further purification of nucleotide pyrophosphatase (Table V). It appears likely that thiamine pyrophosphate splitting is a property of nucleotide pyrophosphatase, although the evidence is more limited than in the case of ATP splitting.

TABLE VI

K_I of ATP for Nucleotide Pyrophosphatase

The incubation mixture (1.0 cc.) contained $0.88 \mu\text{M}$ of DPN, 0.20 cc. of 0.5 M phosphate buffer, pH 7.0, ATP as indicated below, and 0.5 γ of purified enzyme. After 20 minutes at 38° , 0.2 cc. aliquots were analyzed for DPN. $K_s = 1.5 \times 10^{-4}$. $V_{\max} = (K_{sp})/(S) + v = 3.93 \times 10^3$ units per cc. $K_I = K_s I / [(V_{\max} S/v) - S - K_s]$.

ATP, $\mu \times 10^3$	0	5	10	20
DPN splitting, units per cc.....	3360	2640	2280	1560
K_I , moles per l. $\times 10^3$		2.7	3.1	2.5

DPN in an equimolar ratio (4×10^{-3} M) completely inhibited thiamine pyrophosphate splitting by the purified enzyme. Under identical conditions, thiamine pyrophosphate splitting by the crude enzyme was unaltered. The K_s values for thiamine pyrophosphate splitting by the crude and purified enzymes as derived from Fig. 1, *f* are 1.7×10^{-3} and 2.6×10^{-3} mole per liter. The significance of this small difference is questionable.

Other Phosphatase Activities—The purified preparations still contained detectable amounts of phosphatase activity toward several phosphate esters (Table VII). However, these activities were very low, splitting of inorganic pyrophosphate being only 3 per cent as active and the other activities being less than 1 per cent of the rate for DPN splitting. There was no indication during the course of purification that any of these activities became stabilized with reference to DPN splitting, as was the case with adenyly pyrophosphate and thiamine pyrophosphate splitting. It was impossible to determine whether DPN inhibited these phosphatase activities, since the large amounts of enzyme required in the test destroyed the

DPN too rapidly. On the other hand, as previously mentioned, these esters (except for adenosine-5-phosphate) when present in a molar ratio of 25 or greater (with respect to DPN) showed no inhibitory effect on DPN splitting.

TABLE VII

Nucleotide Pyrophosphatase and Other Phosphatase Activities at Different Stages of Purification

The values are ratios of the DPN-splitting activity to the activity in the splitting of the compounds indicated.

Specific activity for DPN splitting, units per mg. protein	Inorganic pyrophosphate	Adenosine-5-phosphate	Adenosine-3-phosphate	NMN	Glucose-6-phosphate	Glycero-phosphate
3*	0.13	0.07	0.09	0.30	0.19	0.13
5†	0.09	0.14	0.14	0.75	0.60	0.29
16	0.21	0.93	1.8	2.2	1.7	1.0
80	1.2	6.5	7.7	10.7	7.5	5.0
162	7.1	20	31	33	40	10.9
1665	26	106	162	230	184	309
2200	34	124	116	110	154	124

* Aqueous potato extract.

† Ammonium sulfate fraction.

TABLE VIII

Effects of Mg^{++} , Ca^{++} , and F^- on Nucleotide Pyrophosphatase

Assays with purified enzyme. The values are given as per cent of the activity observed without metal or fluoride.

Substrate	Mg^{++} , 0.01 M	Ca^{++} , 0.01 M	NaF, 0.1 M	
			Glycylglycine	Phosphate, 0.05 M, pH 7.4
DPN.....	96	87	93	57
TPN.....	53	86	60	
FAD.....	119		185	35
ADP.....	90	110	22	
ATP.....	186	191	55	
" *.....	116	184	27	

* Splitting by crude aqueous extract.

Effects of Metals and Inhibitors—DPN splitting was not stimulated by Mg^{++} or Ca^{++} and was inhibited by F^- only in the presence of phosphate (Table VIII). Other metals (0.01 M) inhibited to a variable extent: Mn^{++} , Co^{++} , Fe^{++} , Cu^{++} , and Al^{+++} inhibited 9, 22, 24, 54, and 59 per cent, respectively. Cyanide and azide at 0.02 M and pH 7.4 did not inhibit. In

Table VIII are shown the effects of Mg^{++} , Ca^{++} , and F^- on the other nucleotide pyrophosphatase activities. The metal activation of ATP splitting by the purified enzyme and the stimulation of FAD splitting by fluoride in the presence of glycylglycine are unexplained.

DISCUSSION

While the present studies have been limited to potatoes as a source material, it appears likely that nucleotide pyrophosphatase occurs in other natural materials as well. Splitting of DPN at the pyrophosphate linkage in rabbit kidney has been reported (4) and the breakdown of TPN⁶ and FAD (27) by animal tissues may on further study be found to involve a similar mechanism. With preparations from brewers' yeast, hydrolytic cleavage of DPN and FAD has been observed (7). As in the present studies, hydrolytic splitting of FAD by these yeast preparations is effectively inhibited by DPN.

The known mechanisms of enzymatic cleavage of the dinucleotides may be summarized as follows: DPN at the nicotinamide-ribose bond (3) and at the pyrophosphate bond by hydrolysis and pyrophosphorolysis (5); TPN at the pyrophosphate bond and at the linkage of the "third" phosphate to the ribose of adenosine; FAD at the pyrophosphate bond by hydrolysis and pyrophosphorolysis (7).

SUMMARY

Nucleotide pyrophosphatase has been purified 750-fold from potatoes. This enzyme splits the pyrophosphate linkages of DPN, DPNH₂, TPN, FAD, ADP, ATP, and thiamine pyrophosphate. Dissociation constants of the enzyme-substrate complexes and some kinetics of inhibition have been studied.

BIBLIOGRAPHY

1. Schlenk, F., *Advances in Enzymol.*, **5**, 207 (1945).
2. Kornberg, A., and Lindberg, O., *J. Biol. Chem.*, **176**, 665 (1948).
3. Handler, P., and Klein, J. R., *J. Biol. Chem.*, **143**, 49 (1942).
4. Kornberg, A., *J. Biol. Chem.*, **174**, 1051 (1948).
5. Kornberg, A., *J. Biol. Chem.*, **176**, 1475 (1948).
6. Kornberg, A., *J. Biol. Chem.*, **182**, 779 (1950).
7. Schrecker, A. W., and Kornberg, A., *J. Biol. Chem.*, **182**, 795 (1950).
8. Lardy, H. A., and Fischer, H. O. L., *J. Biol. Chem.*, **164**, 513 (1946).
9. Fittig, R., and Miller, H. E., *Ann. Chem.*, **255**, 43 (1889).
10. Ochoa, S., *J. Biol. Chem.*, **174**, 133 (1948).
11. Kubowitz, F., and Ott, P., *Biochem. Z.*, **314**, 94 (1943).
12. Lowry, O. H., and Lopez, J. A., *J. Biol. Chem.*, **162**, 421 (1946).

⁶ Kornberg, A., unpublished observations.

13. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
14. Kalckar, H. M., *J. Biol. Chem.*, **167**, 445 (1947).
15. Burch, H. B., Bessey, O. A., and Lowry, O. H., *J. Biol. Chem.*, **175**, 457 (1948).
16. Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **167**, 157 (1947).
17. Weichselbaum, T. G., *Am. J. Clin. Path., Tech. Suppl.*, **7**, 40 (1946).
18. Warburg, O., and Christian, W., *Biochem. Z.*, **310**, 384 (1941-42).
19. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **124**, 397 (1938).
20. Michaelis, L., and Menten, M. L., *Biochem. Z.*, **49**, 333 (1913).
21. von Euler, H., and Adler, E., *Z. physiol. Chem.*, **252**, 41 (1938).
22. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658 (1934).
23. Negelein, E., and Brömel, H., *Biochem. Z.*, **300**, 225 (1939).
24. Warburg, O., and Christian, W., *Biochem. Z.*, **298**, 150 (1938).
25. Kalckar, H. M., *J. Biol. Chem.*, **153**, 355 (1944); **167**, 461 (1947).
26. Krishnan, P. S., *Arch. Biochem.*, **20**, 261 (1949).
27. Comline, R. S., and Whatley, F. R., *Nature*, **161**, 350 (1948).

REVERSIBLE ENZYMATIC SYNTHESIS OF DIPHOSPHOPYRIDINE NUCLEOTIDE AND INORGANIC PYROPHOSPHATE

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Ochoa, Cori, and Cori (1) observed the production of inorganic pyrophosphate (PP) during the oxidation of glutamate, pyruvate, or succinate by dialyzed rat liver dispersions. They were able to show that the PP was not a result of simple hydrolysis of adenosine triphosphate (ATP), since ATP added to these preparations failed to yield PP. The release of PP from ATP by bone and snake venom phosphatases (2) and the occurrence of PP in molds (3) and in yeast (4) have also been described. Recent studies with washed kidney particles (5, 6) have confirmed the production of PP by respiring tissue preparations but have not clarified the mechanism involved.

The mechanism of the biosynthesis of diphosphopyridine nucleotide (DPN) has also remained obscure. The only studies with cell-free systems were reported by Lennerstrand (7), who found that cozymase activity destroyed by incubation with apozymase could be partially restored when active fermentation took place.

An enzyme has now been purified from yeast (8) and liver which catalyzes the reversible synthesis of DPN and PP according to equation (1).



In this report, a study of the purification and properties of the yeast and liver enzymes and of the kinetics of the reaction is presented.

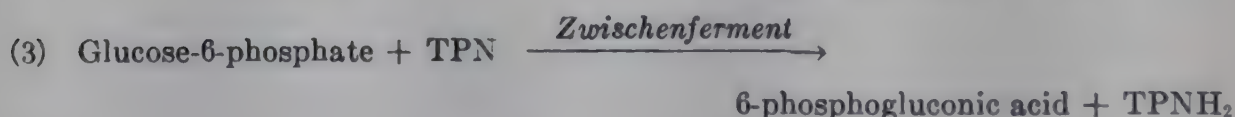
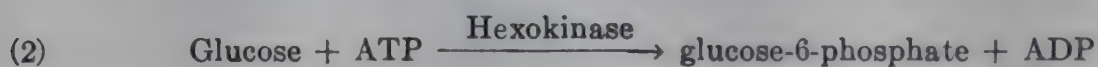
Methods

Materials—ATP was a commercial sample (Sigma Chemical Company) or was prepared by a modification of Lohmann's method (9). Further purification (removal of orthophosphate, adenylate, and adenosine diphosphate) was effected by washing the barium salt five times with cold 0.1 M acetate buffer (pH 4.0). The purity of this preparation is discussed below. Adenosine diphosphate (ADP) and crystalline adenosine-5-phosphate were obtained from the Sigma Chemical Company. DPN of purity 0.72 was prepared according to Williamson and Green (10) with minor modifications. Reduced DPN (DPNH₂) of purity 0.55 was prepared by

¹ This equation may be represented as $\text{NRP} + \text{PPRA} \rightleftharpoons \text{NRPPRA} + \text{PP}$, where N = nicotinamide, R = ribose, P = phosphate, and A = adenine.

Oehlmeier's method (11). Glycylglycine was synthesized by the method of Dunn *et al.* (12). NMN was prepared as previously described (13). Reduced NMN (NMNH₂) was prepared as follows: 15 mg. of DPNH₂ in 1 cc. of 0.25 M glycylglycine buffer (pH 7.4) were incubated for 10 minutes at 38° with 0.2 mg. of purified potato nucleotide pyrophosphatase (13) in a final volume of 1.3 cc. The enzyme was inactivated by adding 0.3 cc. of 2 N NaOH and incubating at 38° for 15 minutes. The conversion of DPNH₂ to NMNH₂ was quantitative, since the optical density at 340 mμ was unaltered and was not decreased by the addition of pyruvate and lactic dehydrogenase. Nicotinamide nucleoside was obtained by hydrolysis of NMN with a nucleotidase purified from potato.² Triphosphopyridine nucleotide (TPN) of 0.55 purity was obtained from liver by a modification³ of the method of Warburg and Christian (14). Flavin-adenine dinucleotide (FAD) is described in a subsequent paper (15). Metaphosphate was purified as follows: 0.2 M barium nitrate was added to 2 volumes of 0.2 M metaphosphoric acid and kept at 0° for 4 hours. The precipitate was washed with cold water and dissolved in dilute HCl. Barium was removed with excess sodium sulfate and the solution neutralized.

Determinations—ATP was determined with the Beckman spectrophotometer by reduction of TPN according to reactions (2) and (3).



The components of the test were as follows: glucose (0.5 M) 0.2 cc., MgCl₂ (0.15 M) 0.1 cc., hexokinase Fraction 3 or 5 (16) (diluted 100-fold) 0.05 cc., *Zwischenferment* (17) (3 mg. per cc.) 0.1 cc., TPN (2 mg. per cc.) 0.1 cc., and water to a final volume of 3.0 cc. The blank absorption cell contained water in place of TPN. Initial readings were obtained and *Zwischenferment* was added to start the reaction. Final and stable readings were reached after about 30 minutes. The proportionality and sensitivity of this method are indicated in Fig. 1. It was essential to exclude in-

² Aqueous potato extract was fractionated with ammonium sulfate between 0.57 and 0.90 saturation. This fraction was dialyzed against water, adjusted to pH 4.5 with acetic acid, and fractionated with ethanol at -5°. The best fraction split orthophosphate from NMN at the rate of 193 μM per hour per mg. of protein at 38° at pH 5.0 in the presence of 0.01 M MgCl₂. The nicotinamide-ribose linkage was not attacked. Compared to the crude extract, the NMN nucleotidase was 30 times purified on the basis of protein concentration and 10 times purified on the basis of adenosine-3-phosphatase activity.

³ Unpublished procedure of Warburg and Christian.

terfering factors which produced large errors. The use of crude hexokinase preparations or of unnecessarily large excesses of purified preparations resulted in low values due to the presence of ATPase and a flavoprotein which reoxidized TPNH_2 . High values were observed with *Zwischenferment* preparations which contained 6-phosphogluconic acid dehydrogenase activity. In some cases this interference was removed by aging the *Zwischenferment* solution 24 hours at 3° .

Preparations of ATP, purified as described above, appeared to be 90 to 100 per cent pure, on the basis of dry weight and the ratios of acid-labile phosphate, acid-stable phosphate, and ultraviolet absorption, but were only about 70 per cent pure when analyzed by the enzymatic method. The analytical data, expressed in micromoles per cc., for a typical ATP solution, were as follows: orthophosphate 0.03, acid-labile phosphate 2.00,

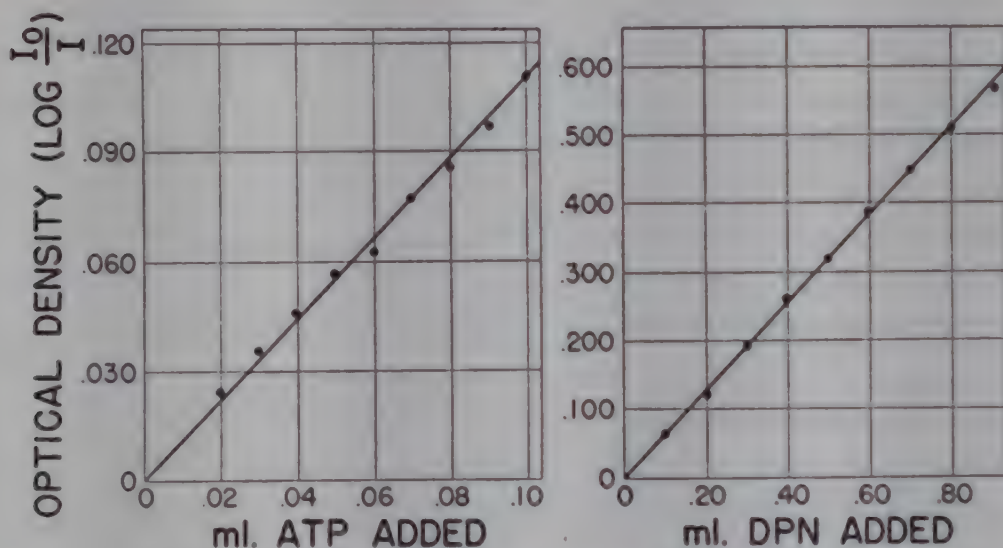


FIG. 1. Determination of ATP and DPN

acid-stable phosphate 1.12, adenine (absorption at $260 \text{ m}\mu$) 1.05, "enzymatic ATP" 0.71. Enzymatic measurement of the ATP produced from DPN in studies of reaction (1) showed a stoichiometric conversion (± 5 per cent), suggesting that the enzyme assay values are correct. The higher chemical assay values for ATP preparations (commercial or prepared in this laboratory) may be due to the presence of very similar adenine nucleotides.

DPN was determined spectrophotometrically by reduction with crystalline alcohol dehydrogenase (18), kindly furnished by Dr. E. Racker or prepared by his simplified method from yeast.⁴ The extinction coefficient of $6.22 \times 10^6 \text{ cm}^2 \times \text{mole}^{-1}$ at $340 \text{ m}\mu$ (19) was used. The components

⁴ The details of this unpublished method were made available through the kindness of Dr. E. Racker.

were ethanol 0.3 cc., glycine (1.5 per cent) 0.2 cc., sodium pyrophosphate buffer (0.03 M, pH 8.5) 1.5 cc., alcohol dehydrogenase 5 to 10 γ , and water to a final volume of 3.0 cc. The blank absorption cell contained water in place of alcohol dehydrogenase. The latter was added to start the reaction after initial readings were obtained. Complete reduction with stable readings was reached after 5 minutes. The data in Fig. 1 illustrate the proportionality and sensitivity of this method. The specificity of the determination was verified frequently by observing the complete removal of the optical density at 340 $m\mu$ on addition of pyruvate and small amounts (0.1 to 0.2 γ) of purified lactic dehydrogenase. DPN was also determined by reduction with the triose phosphate dehydrogenase system as previously described (5). With this method low values were frequently obtained due to the reoxidation of $DPNH_2$ by an α -glycerophosphate dehydrogenase impurity in the oxidizing system.

$DPNH_2$ was determined spectrophotometrically by oxidation with pyruvate and lactic dehydrogenase (20).

PP was determined as orthophosphate (21) after hydrolysis for 15 minutes at 100° in 1 N H_2SO_4 of the washed manganous salt (22). To an incubation mixture (heated at 100° for 1 minute to stop the reaction) containing 0.4 μM (micromole) or more of PP in 0.5 cc. were added 0.2 cc. of 1 M acetate buffer (pH 5.0) and 0.2 cc. of 0.1 M $MnCl_2$. After 15 minutes at room temperature, the flocculent precipitate was centrifuged and washed with 1.0 cc. of 0.01 M $MnCl_2$ and 0.2 cc. of 10 per cent acetone. The precipitate was dissolved in 0.4 cc. of 0.1 N HCl and diluted with water, and aliquots were removed for analysis of orthophosphate, acid-labile phosphate, and for ultraviolet absorption. The conditions of this procedure, which were necessary for the quantitative recovery of PP, resulted in the precipitation of traces of ATP, when ATP was present in concentrations comparable with those of PP. Corrections based on the ultraviolet absorption of ATP were therefore applied. Recoveries of 94 to 108 per cent were obtained with 0.4 to 2.0 μM of PP in 0.5 cc.

TPN and FAD were determined as described in the previous paper (13).

Enzyme Assay—The components of the test were as follows: ATP (0.02 M) 0.1 cc., NMN (0.05 M) 0.05 cc., glycylglycine buffer (0.25 M, pH 7.4) 0.20 cc., $MgCl_2$ (0.15 M) 0.10 cc., enzyme, and water to make a final volume of 1.0 cc. To avoid interference by DPN nucleosidase (23, 5), 0.1 cc. of nicotinamide (2 M) was added in assays of crude liver fractions. After incubation at 38° for 20 minutes, 1.0 cc. of 10 per cent trichloroacetic acid was added. The supernatant solution was neutralized with 2 N NaOH with the aid of an internal indicator (brom thymol blue or phenol red), and 1.0 cc. was pipetted into each of two absorption cells for DPN analysis. With the purified yeast and liver enzymes, the use of trichloroacetic

acid was unnecessary and analyses were performed directly on aliquots of the incubation mixtures.

A unit of enzyme activity was defined as the amount causing the synthesis of 1 μ M of DPN per hour, and specific activity as units per mg. of protein. Proportionality to enzyme concentration was observed in this test with crude as well as purified preparations when 1 unit or less was present in the test system.

Protein concentration was determined by the nephelometric method of Bücher (24) with the Beckman spectrophotometer at 340 m μ .

Results

Purification of Yeast and Liver Enzymes

Purification of Yeast Enzyme—Beer or ale yeast was washed with tap water by sedimentation at 3°, pressed, and dried at 23°. An autolysate was prepared by suspending the dry yeast in 3 volumes of a liquid medium at 38°. With water, only traces of enzyme activity were obtained. With 0.1 M potassium phosphate buffer (pH 7.4) or with 0.1 M sodium bicarbonate (saturated with 5 per cent CO₂ at 23°), the autolysates were highly active. The yield and specific activity of enzyme obtained with bicarbonate were somewhat greater than with phosphate. While all the bicarbonate autolysates of seven different yeasts tested were active, there was a wide variation in the yield and specific activity of enzyme. The optimal autolysis time varied from 20 to 48 hours for different batches of a given yeast and had to be determined separately for each batch. Fig. 2 illustrates the course of extraction of enzyme activity and protein from a sample of top ale yeast,⁵ the further purification of which is described below. Assays were on material carried through the first step of purification (ammonium sulfate). Assays of the crude autolysate were much less reliable but showed the same general pattern described in Fig. 2. This prolonged and extensive autolysis increased the specific activity of the enzyme preparation approximately 10-fold with relatively little sacrifice in yield.

100 gm. of dry top ale yeast were suspended in 300 cc. of 0.1 M sodium bicarbonate (saturated with 5 per cent CO₂ at 23°) and maintained at 38° for 48 hours. The autolysis mixture after centrifugation yielded a clear, amber supernatant (autolysate, Table I), to which was added an equal volume of 0.1 M sodium bicarbonate (saturated with 5 per cent CO₂ at 23°). All subsequent operations were carried out at 3°. 136 gm. of ammonium sulfate were added and after 10 minutes the small precipitate was collected by centrifugation and dissolved in water (ammonium sulfate,

⁵ Kindly furnished by Mr. F. X. Schneider of the Gunther Brewing Company, Baltimore.

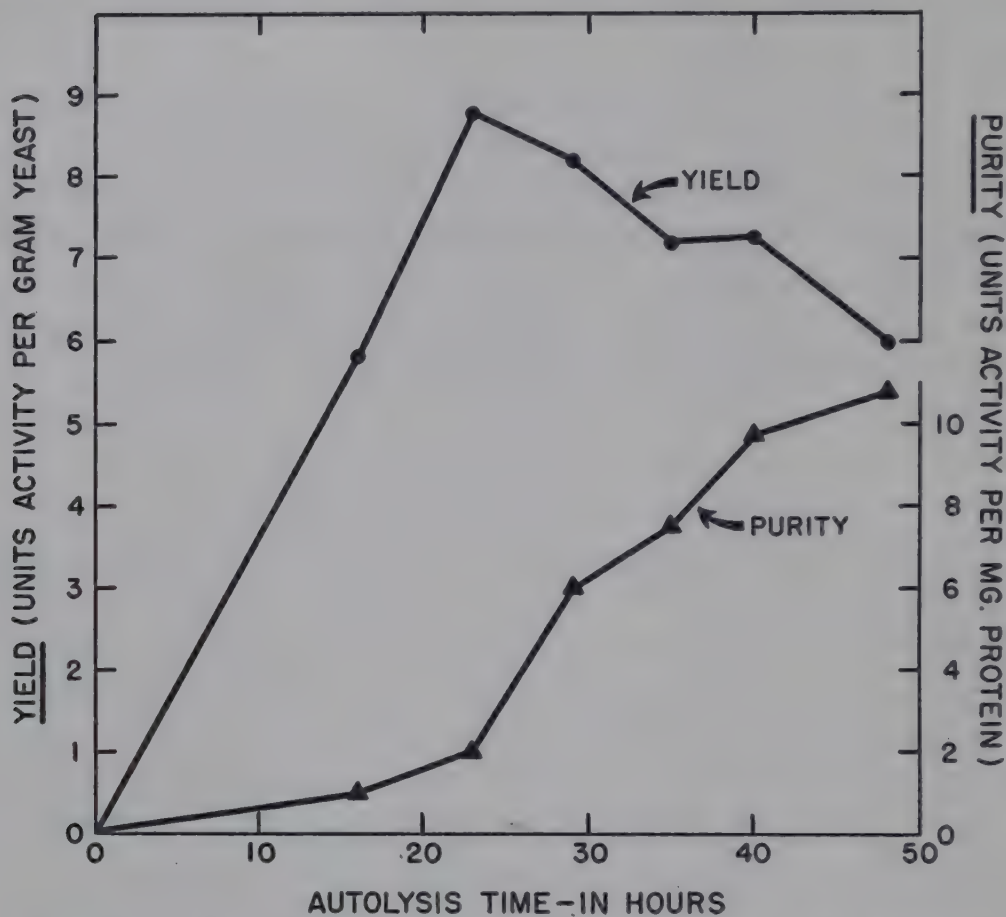


FIG. 2. Effect of autolysis on the yield and purity of the yeast enzyme. Assays were on material carried through the first step of purification (ammonium sulfate, Table I).

TABLE I
Purification of Yeast and Liver Enzymes

	Step	Volume of fraction	Total activity	Over-all yield	Specific activity
		cc.	units	per cent	units per mg. protein
Yeast	Autolysate (48 hrs., 38°)	210	716		0.24
	Ammonium sulfate	30	700	98	14.5
	“ “ refractionated	6	417	58	124
	Ppt. at pH 4.8	5	313	44	453
Liver	Extract	80	202		0.11
	Ammonium sulfate	13	163	81	0.71
	Adsorption, ammonium sulfate	4	57	28	12.4

Table I). Overnight a flocculent precipitate formed which was centrifuged off and discarded. To this solution were added 7.9 gm. of ammonium sulfate. After 10 minutes the precipitate was centrifuged off and dissolved in water. The slightly turbid solution (ammonium sulfate, refractionated)

could be partially clarified by centrifugation (at 10,000 R.P.M. in an angle centrifuge for 5 minutes) without loss of activity. An equal volume of 0.1 M acetate buffer (pH 4.80) was added to the supernatant and after 10 minutes this faintly turbid solution was centrifuged at 10,000 R.P.M. for 7 minutes in an angle centrifuge. The precipitate was dissolved in 0.1 M glycylglycine buffer, pH 7.4 (precipitate at pH 4.8). This fraction (referred to as purified yeast enzyme) represented a yield of 44 per cent of the activity in the 48 hour autolysate. On the basis of protein content it is approximately 2000 times more pure than the 48 hour autolysate and over 10,000 times more pure than the 23 hour autolysate.

When stored at 3° for 2 weeks, there was a loss of about 25 per cent in activity of the enzyme. There was a 44 per cent loss in activity when the enzyme, diluted 10-fold, was incubated for 1 hour at 38°; crystalline bovine albumin (1 mg. per cc.) did not prevent this inactivation.

Purification of Liver Enzyme—Homogenates of rat liver and brain carried out DPN synthesis from NMN and ATP in the presence of nicotinamide. A convenient source of the enzyme was hog liver, from which active, stable acetone powders were prepared. Fresh hog liver (100 gm.) was homogenized in acetone (−10°, 500 cc.) in a Waring blender. The residue collected on a Büchner funnel was resuspended in cold acetone, filtered off, and dried at room temperature. 10 gm. of powder were extracted with 100 cc. of 0.1 M Na_2HPO_4 for 10 minutes at room temperature. Subsequent operations were at 3° unless otherwise indicated. The residue was separated by centrifugation and discarded. To the extract (Table I), were added 16 gm. of ammonium sulfate; the precipitate was removed by centrifugation and discarded. 8 gm. of ammonium sulfate were added to the supernatant, and the precipitate collected by centrifugation was dissolved in water to a volume of 40 cc. This fraction was reprecipitated by adding 8 gm. of ammonium sulfate, centrifuging, and dissolving the resulting precipitate in water (ammonium sulfate, Table I). This fraction was diluted with water (23°) to 94 cc. and adsorbed on 9.4 cc. (75 mg.) of calcium phosphate gel (13) during a 5 minute period. The gel was washed three times with 36 cc. of cold 0.02 M phosphate buffer (pH 7.0) and eluted with 37 cc. of cold 0.5 M K_2HPO_4 . To the clear eluate were added 6.3 gm. of ammonium sulfate; the precipitate was collected by centrifugation and dissolved in water (adsorption, ammonium sulfate, Table I). This fraction, hereafter referred to as purified liver enzyme, was 100 times more active than the acetone powder extract on the basis of protein content and represented a yield of 28 per cent. The stability of this preparation was greater than that of the purified yeast enzyme; no significant loss of activity was detected during storage at 3° for 1 to 2 weeks.

Properties of Enzymes

Equilibrium—The crude yeast and liver preparations contained phosphatase and other interfering activities (DPN nucleosidase in liver) which prevented a study of reaction (1) and its equilibrium. For example, nucleotide pyrophosphatase (13) in the first yeast ammonium sulfate fraction split added DPN in the absence of inorganic pyrophosphate at a very rapid rate. Fortunately, the large concentration of ATP in the assay for DPN synthesis from NMN and ATP inhibited DPN hydrolysis by nucleotide pyrophosphatase and thus permitted the accumulation of DPN. In the purified enzyme preparations, all interfering activities were absent. Fig. 3 and Table II indicate the attainment of stable equilibria with the

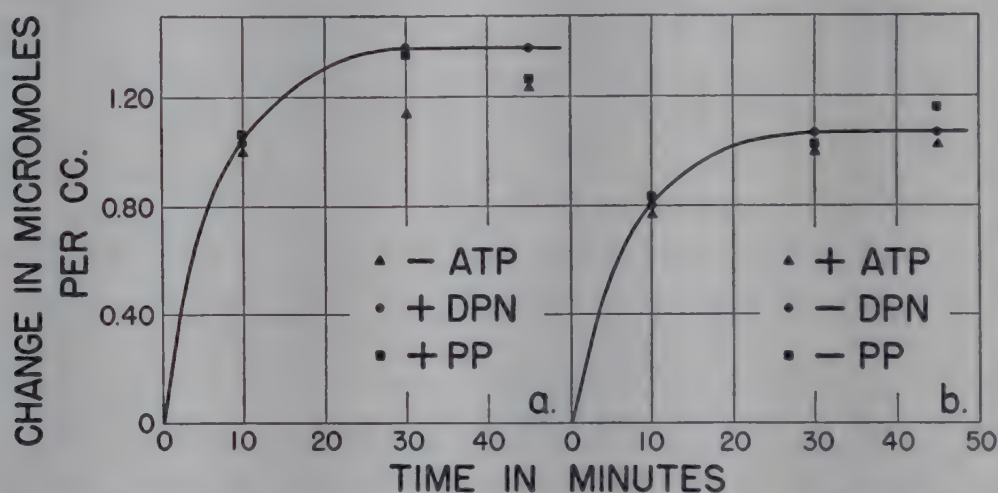


FIG. 3. Attainment of equilibrium with yeast enzyme. (a) NMN + ATP, (b) DPN + PP. At 0 minute, 1.0 cc. of reaction mixture contained (a) 2.78 μM of NMN, 4.05 μM of ATP, 3 μM of MgCl_2 , 100 μM of glycylglycine buffer, pH 7.4, and 0.025 mg. of purified yeast enzyme; (b) 1.86 μM of DPN, 2.00 μM of PP, 0.5 μM of MgCl_2 , 50 μM of glycylglycine buffer, pH 7.4, and 0.017 mg. of purified yeast enzyme. At 45 minutes, the ATP, DPN, and PP concentrations, in μM per cc., were respectively, (a) 2.81, 1.38, and 1.26; (b) 1.02, 0.79, and 0.84.

purified yeast and liver enzymes, starting either with NMN and ATP or with DPN and PP. Emphasis has been given to the DPN values, since these are considered most precise. The equilibrium constant, $K = (\text{DPN})(\text{PP})/(\text{NMN})(\text{ATP})$, calculated from these data is approximately 0.45. The discrepancies between the several determinations of K may in part have been due to the formation of complexes of PP with Mg^{++} and protein.

Nature of Reaction—As previously reported (8), the values for acid-labile phosphate were unchanged during the course of the reaction and no inorganic orthophosphate was produced. From the known specificities of alcohol dehydrogenase (18, 19), D-glyceraldehyde-3-phosphate dehydrogenase (25), and hexokinase (16), the identity of the formed DPN and ATP

appears certain. No specific test is available for NMN. Although metaphosphate did not react with DPN (see below), it was considered desirable to differentiate in a more positive way the formed PP from metaphosphate. A partially purified yeast inorganic pyrophosphatase (26), free of metaphosphatase, was used to determine PP. In an experiment with purified liver enzyme in which $0.56\ \mu\text{M}$ of DPN was formed, the addition of inorganic pyrophosphatase at the conclusion of the experiment released $1.14\ \mu\text{M}$ of inorganic orthophosphate, indicating the presence of $0.57\ \mu\text{M}$ of PP. In a control experiment, $0.44\ \mu\text{M}$ of PP was added to an incubation mixture treated identically, except that the liver enzyme was first inactivated by heating; $0.42\ \mu\text{M}$ of PP was recovered.

TABLE II

Attainment of Equilibrium with Liver Enzyme

The incubation mixture (1.0 cc.) contained $3\ \mu\text{M}$ of MgCl_2 , $60\ \mu\text{M}$ of glycylglycine buffer, pH 7.4, and 0.2 mg. of purified liver enzyme. For DPN synthesis, $2.70\ \mu\text{M}$ of NMN and $1.80\ \mu\text{M}$ of ATP were present, and for DPN breakdown, $2.00\ \mu\text{M}$ each of DPN and PP.

		Changes				$K = \frac{(\text{DPN})(\text{PP})}{(\text{NMN})(\text{ATP})}$
		15 min.	30 min.	45 min.	60 min.	
		μM	μM	μM	μM	
DPN synthesis	ATP	-0.44	-0.58	-0.64	-0.75	0.27
	DPN	+0.45	+0.61	+0.70	+0.74	
	Yeast enzyme*					0.44
DPN breakdown	ATP	+0.76	+0.95	+0.99	+1.03	0.46
	DPN	-0.97	-1.13	-1.19	-1.19	
	" (no PP)†	-0.01	0.00	-0.01	0.00	0.61
	Yeast enzyme*					

* From Fig. 3.

† PP omitted from the incubation mixture.

Reaction with Reduced Nucleotides— NMNH_2 and DPNH_2 replaced the corresponding oxidized nucleotides as formulated in reaction (4).



In studies with the purified yeast enzyme, the production of DPNH_2 from NMNH_2 required the presence of ATP and approached an equilibrium as shown in Table III. The splitting of DPNH_2 required the presence of PP and resulted in the appearance of an equivalent amount of ATP. The rate of DPNH_2 splitting was 10.7 units per cc. as compared with 38.0 units per cc. for DPN breakdown.

Specificity—DPN was not synthesized by the purified yeast enzyme when NMN was replaced by nicotinamide nucleoside or when ADP or adenosine-

5-phosphate was substituted for ATP. TPN and flavin-adenine dinucleotide did not react. With the purified liver enzyme, DPN breakdown did not occur when PP was replaced by inorganic orthophosphate or metaphosphate.

Effects of Mg^{++} and Mn^{++} —The Mg^{++} requirement of the yeast and liver enzymes is shown in Fig. 4. The Michaelis constants (K_s) (27) for Mg^{++} estimated from these curves were approximately 5×10^{-4} and 2×10^{-4} mole per liter for the yeast and liver enzymes, respectively. Stimulation by Mn^{++} was relatively small and limited.

Effects of Other Metals, Fluoride, and Dinitrophenol—The rate of DPN synthesis by the yeast enzyme in 0.1 M glycylglycine buffer, pH 7.4, was 20.3 units per cc. as compared with a rate of 7.6 units per cc. in barbital

TABLE III
Reaction with Reduced Nucleotides

The incubation mixture (1.0 cc.) contained, for $DPNH_2$ synthesis, 2.92 μM of ATP, 1.18 μM of $NMNH_2$, 2.0 μM of $MgCl_2$, 70 μM of glycylglycine buffer, pH 7.4, and 0.07 mg. of purified yeast enzyme; for $DPNH_2$ breakdown, 1.64 μM of $DPNH_2$, 2.85 μM of PP, 1.0 μM of $MgCl_2$, and buffer and enzyme as above.

		Changes, μM			
		30 min.	60 min.	120 min.	180 min.
DPNH ₂ synthesis	DPNH ₂	+0.38	+0.52	+0.70	+0.73
	" (no ATP)*	+0.05	+0.02	+0.04	
" breakdown	DPNH ₂	-0.46	-0.59	-0.56	-0.56
	" (no PP)†		-0.05		
	ATP		+0.56		

* ATP omitted from the incubation mixture.

† PP omitted from the incubation mixture.

buffer (Fig. 4, a). Barbital was not inhibitory when added to glycylglycine buffer, suggesting that the more rapid rate with glycylglycine was due to removal of a metallic inhibitor. Consistent with this possibility was the observation that 1 mg. per cc. of a commercial ATP preparation (other than Sigma) inhibited the yeast enzyme by 70 per cent and that this inhibition remained after ashing. The decreases in rate produced by Fe^{++} , Cu^{++} , and Zn^{++} at 1×10^{-3} M were only 20 to 50 per cent and therefore do not implicate any of these metals as a likely inhibitor. 2,4-Dinitrophenol (1×10^{-4} M) did not inhibit the yeast enzyme, nor did fluoride (5×10^{-2} M) inhibit the liver enzyme.

Substrate Affinities and Maximal Rates—The dependence of reaction rates on substrate concentration is shown in Fig. 5. Each substrate was tested in the presence of an optimal concentration of the corresponding re-

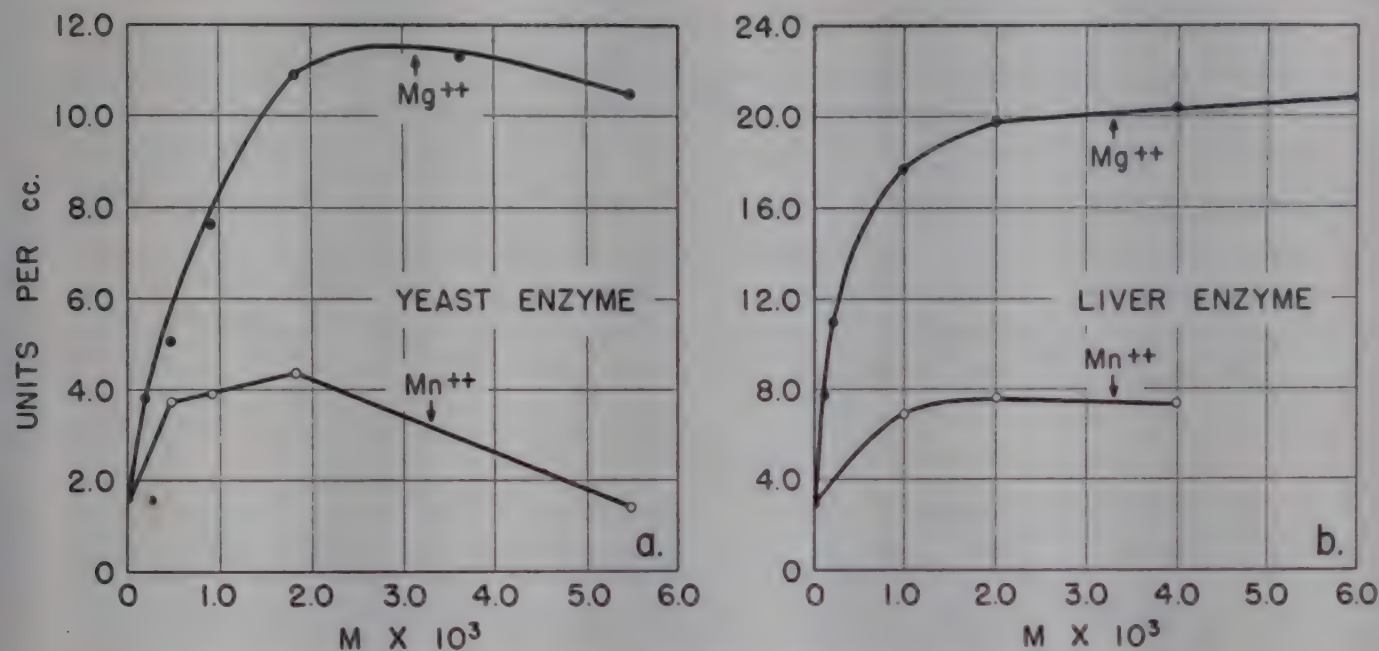


FIG. 4. Effects of Mg^{++} and Mn^{++} on the yeast and liver enzymes. (a) purified yeast enzyme (0.75 γ); 0.3 cc. of barbital buffer (0.1 M, pH 7.4) replaced glycylglycine; (b) purified liver enzyme (80 γ); 0.1 cc. of phosphate buffer (0.5 M, pH 7.0) replaced glycylglycine.

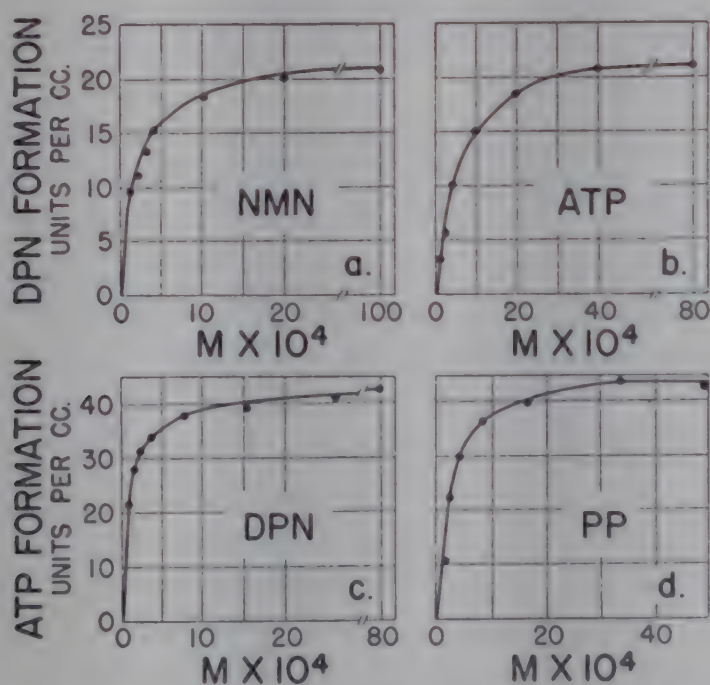


FIG. 5. Substrate affinities of NMN, ATP, DPN, and PP for the liver enzyme. The incubation mixture (0.5 cc.) contained 2 μM of $MgCl_2$ in (a) and (b) and 0.5 μM in (c) and (d), 25 μM of phosphate buffer, pH 7.0, and 0.01 mg. of purified liver enzyme. The concentrations of reactants were 8×10^{-3} M ATP in (a), 1×10^{-2} M NMN in (b), 3.4×10^{-3} M PP in (c), and 2.6×10^{-3} M DPN in (d). Incubation was for 5 minutes at 38° directly in absorption cells. The reaction was stopped by a 30 second immersion in boiling water. DPN analyses were performed in (a) and (b) and ATP analyses in (c) and (d).

actant. The dissociation constants of the liver enzyme-substrate complexes (27) calculated from the values were as follows, in moles per liter: 1.5×10^{-4} for NMN, 4.6×10^{-4} for ATP, 0.83×10^{-4} for DPN, and 1.9×10^{-4} for PP. With use of the optimal substrate concentrations, values for the maximal reaction velocities were obtained. The ratio of $V_{\max.}$ for DPN synthesis to $V_{\max.}$ for DPN breakdown was 0.48 for the purified liver enzyme and 0.42 for the purified yeast enzyme. The ratio was 0.39 for the same yeast enzyme when tested after 6 weeks storage, at which time it had lost 86 per cent of its activity.

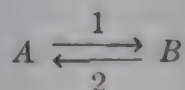
DISCUSSION

The known enzymatic reactions of ATP involve either a hydrolytic cleavage of one of the pyrophosphate linkages or a transfer of the terminal phosphate group to an acceptor molecule such as creatine, adenylic acid, or a sugar. The condensation of ATP with a mononucleotide to split out PP and form a dinucleotide has not been observed previously. The reverse reaction, namely the cleavage of a dinucleotide by PP, resembles the phosphorolytic splitting of glycogen, disaccharides, and nucleosides and by analogy may be termed "pyrophosphorolytic." The equilibrium constant of this reaction is approximately 0.4, a value consistent with the exchange of the pyrophosphate bond of DPN and that of PP for the two pyrophosphate bonds of ATP.

The enzyme for this reaction has been purified over 10,000-fold from yeast autolysates, but there is no indication that purity has been approached. Even with a 30 per cent yield (based on the most active autolysates) at this stage, only 1 mg. of protein was obtained from 100 gm. of dry yeast. Efforts at further purification therefore would require much larger amounts of source material than are now readily available. Thus far, there have been no indications that more than one enzyme or that a coenzyme (other than Mg^{++}) is required. Purification of the liver enzyme has been carried only far enough to permit studies of the reaction equilibrium. While a careful comparison of the yeast and liver enzymes has not been made, available data suggest a close similarity. Some observations in this report are limited to one of the two enzymes; choice of the liver or yeast enzyme for an experiment was determined only by convenience.

The apparent concentration of the enzyme in hog liver acetone powder is sufficient at optimal substrate concentrations to synthesize the estimated DPN content of liver (28) in less than 5 minutes. The ATP concentration in liver (rat) is 1.5×10^{-3} M (29), a value nearly maximal for DPN synthesis (Fig. 5, b). Therefore, if other possible controlling factors are neglected, the concentration of NMN would govern the rate of DPN synthesis. Similar considerations seem to apply to yeast as well.

It is of some interest to know whether, in an enzyme-catalyzed reaction, the equilibrium constant can be calculated from the velocities of the opposing reactions. An expression derived from kinetic⁶ and thermodynamic⁷ considerations for reaction



is

$$(5) \quad K_{eq.} = \frac{V_{1\max.}}{V_{2\max.}} \times \frac{K_B}{K_A}$$

where $K_{eq.}$ is the equilibrium constant, B/A , $V_{\max.}$ is the maximal velocity, and K_A and K_B are the respective affinity constants. Substitution of the experimentally obtained values in equation (5) gives the following result:

$$K_{eq.} = 0.48 \times \frac{(0.83 \times 10^{-4})(1.9 \times 10^{-4})}{(1.5 \times 10^{-4})(4.6 \times 10^{-4})} = 0.12$$

The value of 0.12 is to be compared with the experimental values of 0.27 to 0.61. A similar substitution of Bücher's data (24) for the system

(6) D-1,3-Diphosphoglyceric acid + ADP \rightleftharpoons D-3-phosphoglyceric acid + ATP
in equation (5) is as follows:

$$K_{eq.} = \frac{3.2 \times 10^8}{3.6 \times 10^4} \times \frac{(2 \times 10^{-7})(1.1 \times 10^{-7})}{(1.8 \times 10^{-9})(2 \times 10^{-7})} = 0.54 \times 10^3$$

The experimental values for $K_{eq.}$ were in the range 2.9 to 3.5×10^3 . Thus there is a 6-fold discrepancy with Bücher's data and a 4-fold one with ours. While the basis for these discrepancies is not clear, it may be noted that such deviations in the calculated result are readily produced from rather small errors in experimental values. Therefore, it would seem desirable in further tests of equation (5) to consider the use of simpler systems such as the fumarate-malate or glucose-1-phosphate-glucose-6-phosphate equilibria.

The synthesis of PP in reaction (1) and in the analogous reaction with FAD (15) makes it probable that the accumulation of PP in tissues and in fungi may be explained by a sequence of three reactions as represented in Fig. 6. (a) The irreversible hydrolysis of DPN or FAD by nucleotide pyrophosphatase yields NMN or flavin mononucleotide and adenosine-5-phosphate. This enzyme occurs in potatoes (13), yeast (15), kidney (5),

⁶ By Dr. B. L. Horecker.

⁷ By Dr. J. Gergely.

and very likely in liver.⁸ (b) Adenosine-5-phosphate is phosphorylated to ATP in the course of fermentation or respiration. (c) NMN or flavin mononucleotide combines with ATP to regenerate DPN or FAD (thus permitting catalytic participation of the dinucleotides) and to produce PP. An important controlling factor in the accumulation of PP is the rate of its enzymatic hydrolysis, a reaction which is potent and wide-spread in nature.

SUMMARY

1. An enzyme, purified from autolysates of ale yeast and from extracts of hog liver acetone powder, catalyzes the following reaction: nicotinamide mononucleotide + ATP \rightleftharpoons DPN + inorganic pyrophosphate.

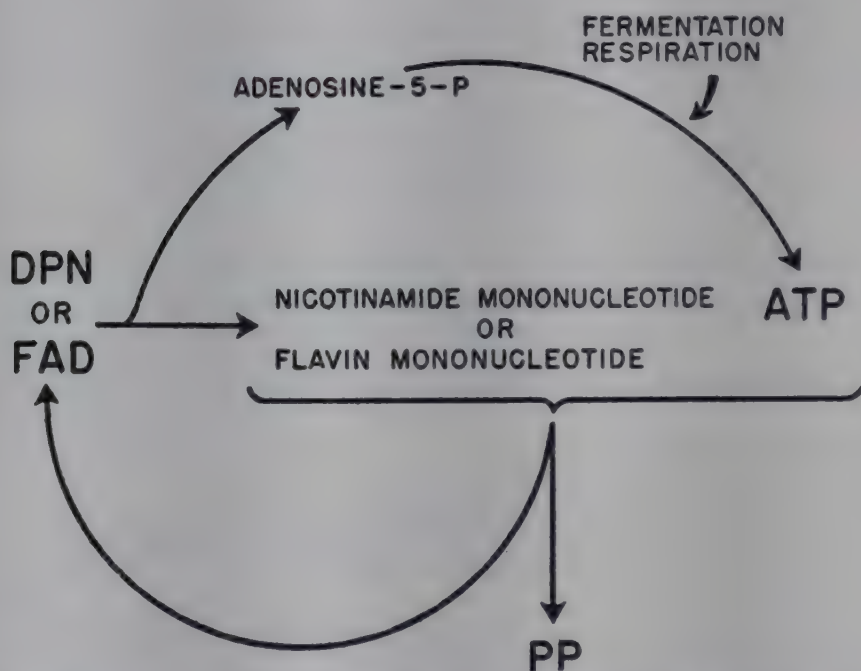


FIG. 6. Proposed mechanism of inorganic pyrophosphate accumulation in tissues and fungi.

2. The enzyme is specific with respect to each of the reactants except that reduced nicotinamide mononucleotide and DPNH_2 may be substituted for the corresponding oxidized nucleotides.

3. Mg^{++} is required and cannot be replaced by Mn^{++} .

4. The equilibrium constant is approximately 0.45. The substrate affinity constants and maximal reaction rates were determined and their relation to the equilibrium constant is discussed.

⁸ DPN (0.002 M) was incubated with rat liver homogenate (150 mg.) in the presence of Mg^{++} (0.006 M) and nicotinamide (0.2 M) in 1.0 cc. of 0.03 M phosphate buffer, pH 7.7. Samples were analyzed for DPN and nicotinamide-ribose (N-R) moiety (30). After 1 hour at 38°, 57 per cent of the DPN and 76 per cent of the N-R remained; after 2 hours, 33 per cent of the DPN and 61 per cent of the N-R remained. Therefore, cleavage of DPN in which the N-R linkage remained intact is indicated.

5. A mechanism for the accumulation of inorganic pyrophosphate in tissues and in fungi is presented.

BIBLIOGRAPHY

1. Cori, C. F., in A symposium on respiratory enzymes, Madison (1942).
2. Hitchings, G. H., and Fuller, H. S., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **128**, p. xlv (1939).
3. Mann, T., *Biochem. J.*, **38**, 345 (1944).
4. Umschweif, B., and Gibaylo, K., *Acta biol. exp.*, Warsaw, **11**, 6 (1937). Lindahl, P. E., and Lindberg, O., *Nature*, **157**, 335 (1946). Juni, E., Kamen, M. D., Reiner, J. M., and Spiegelman, S., *Arch. Biochem.*, **18**, 387 (1948).
5. Kornberg, A., and Lindberg, O., *J. Biol. Chem.*, **176**, 665 (1948).
6. Cross, R. J., Taggart, J. V., Covo, G. A., and Green, D. E., *J. Biol. Chem.*, **177**, 655 (1949).
7. Lennerstrand, A., *Ark. Kemi, Mineral. o. Geol.*, **14 A**, No. 16 (1941).
8. Kornberg, A., *J. Biol. Chem.*, **176**, 1475 (1948).
9. Lohmann, K., *Biochem. Z.*, **254**, 381 (1932).
10. Williamson, S., and Green, D. E., *J. Biol. Chem.*, **135**, 345 (1940).
11. Ohlmeyer, P., *Biochem. Z.*, **297**, 66 (1938).
12. Dunn, M. S., Butler, A. W., and Deakers, T., *J. Biol. Chem.*, **99**, 217 (1932-33).
13. Kornberg, A., and Pricer, W. E., Jr., *J. Biol. Chem.*, **182**, 763 (1950).
14. Warburg, O., and Christian, W., *Biochem. Z.*, **287**, 291 (1936).
15. Schrecker, A. W., and Kornberg, A., *J. Biol. Chem.*, **182**, 795 (1950).
16. Berger, L., Slein, M. W., Colowick, S. P., and Cori, C. F., *J. Gen. Physiol.*, **29**, 379 (1946).
17. Warburg, O., and Christian, W., *Biochem. Z.*, **254**, 438 (1932).
18. Negelein, E., and Wulff, H.-J., *Biochem. Z.*, **293**, 351 (1937).
19. Horecker, B. L., and Kornberg, A., *J. Biol. Chem.*, **175**, 385 (1948).
20. Kubowitz, F., and Ott, P., *Biochem. Z.*, **314**, 94 (1943).
21. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
22. Jones, L. T., *Ind. and Eng. Chem., Anal. Ed.*, **14**, 536 (1942).
23. Handler, P., and Klein, J. R., *J. Biol. Chem.*, **143**, 49 (1942).
24. Bücher, T., *Biochim. et biophys. acta*, **1**, 292 (1947).
25. Mehler, A. H., Kornberg, A., Grisolia, S., and Ochoa, S., *J. Biol. Chem.*, **174**, 961 (1948).
26. Bailey, K., and Webb, E. C., *Biochem. J.*, **38**, 394 (1944).
27. Michaelis, L., and Menten, M. L., *Biochem. Z.*, **49**, 333 (1913).
28. Schlenk, F., in A symposium on respiratory enzymes, Madison (1942).
29. Rapoport, S., *J. Biol. Chem.*, **161**, 429 (1945).
30. Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **167**, 157 (1947).

REVERSIBLE ENZYMATIC SYNTHESIS OF FLAVIN-ADENINE DINUCLEOTIDE

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The synthesis of flavin-adenine dinucleotide (FAD) from riboflavin by human blood cells *in vivo* and *in vitro* was first observed by Klein and Kohn (1). Trufanov (2) reported the formation of FAD when riboflavin was incubated with rat tissue slices. He postulated a condensation of riboflavin with adenosine diphosphate (ADP) and considered the maintenance of cell structure essential for the reaction. The reversible synthesis of diphosphopyridine nucleotide (DPN) from nicotinamide mononucleotide (NMN) and adenosine triphosphate (ATP) with the simultaneous formation of inorganic pyrophosphate (PP) by enzymes in yeast and liver (3, 4) suggested the possibility that FAD might be formed by an analogous reaction from riboflavin phosphate (flavin mononucleotide, FMN) and ATP:



In the present paper are described the partial purification of an enzyme from yeast that catalyzes the reversible reaction (1) and some of the conditions which influence this reaction.

Methods

Materials—FAD¹ was prepared as the barium salt from fresh bakers' yeast by the method of Warburg and Christian (5). Solutions were assayed for total flavin content by measuring the absorption at 450 m μ with a model DU Beckman spectrophotometer, with use of the absorption coefficient of 1.13×10^7 cm.² mole⁻¹ (5), and for FAD content by the manometric method (5). Results by both methods were in agreement with a purity of about 90 per cent. FMN was prepared from FAD by hydrolysis with potato nucleotide pyrophosphatase (6) as follows: A solution containing 0.2 μ M (micromole) of FAD, 0.5 ml. of 0.5 M phosphate buffer (pH 7.5), and 0.015 mg. of purified nucleotide pyrophosphatase was incubated at 37°. After 25 minutes it was immersed in boiling water for 3 minutes, cooled, and diluted with water to 2 ml. The solution was free of FAD, as shown by the manometric test. ATP, ADP, adenosine-5-phosphate, and DPN were as previously described (4), and were used as neutralized solutions. D-Amino acid oxidase free of FAD was prepared as a lyophilized powder (5).

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¹ Kindly furnished by Dr. L. A. Heppel.

Determinations—FAD was determined by the method of Warburg and Christian (5), which involves the measurement of oxygen consumption as a function of FAD concentration in the oxidation of DL-alanine by D-amino acid oxidase.

Conversion of FMN to FAD (Enzyme Assay)—The incubation mixture contained 3 to 10 units of enzyme, 7.5 μM of MgCl_2 , 2 μM of ATP, 20 $\text{m}\mu\text{M}$ of FMN, and 25 μM of phosphate buffer (pH 7.5) in a final volume of 1.0 ml. After incubation for 6 to 15 minutes at 37°, the mixture was immersed in boiling water for 3 minutes, cooled, and centrifuged. An aliquot of the supernatant was assayed for FAD by the D-amino acid oxidase test. A unit of enzyme activity was defined as the amount causing the synthesis of 1 $\text{m}\mu\text{M}$ of FAD per hour under the conditions of the assay, and specific activity as units per mg. of protein. The rate of FAD formation was

TABLE I
Purification of Enzyme

Fraction	Volume	Total activity	Yield	Specific activity
	ml.	units	per cent	units per mg. protein
Autolysate, from 100 gm. dried yeast.....	162	4340		0.91
I. Ammonium sulfate.....	66	7070	163	7.4
II. Ethanol.....	48	4060	57	20.2
III. Aluminum hydroxide gel eluate.....	41	3480	86	30.2
IV. Ethanol.....	26	3340	96	50.7
V. Calcium phosphate gel eluate.....	17	1970*	59	83.0

* In repeated preparations, the total activity of Fraction V varied between 1600 and 2800 units and the specific activity between 51 and 86 units per mg.

roughly proportional to the enzyme concentration. Thus, with 0.156, 0.078, and 0.039 mg. of protein (Fraction V, Table I), specific activities of 64, 66, and 73 units per mg., respectively, were found; similar results were obtained with crude fractions.

FAD breakdown was determined by the increase in fluorescence according to the method of Burch, Bessey, and Lowry (7) with a model 12-A Coleman photofluorometer. A mixture containing 30 to 40 units of enzyme, 3.5 μM of phosphate buffer (pH 7.7), 1 μM of MgCl_2 , 1 to 1.5 μM of DPN, 30 to 35 μM of NaF, varying amounts of PP, and 5 to 15 $\text{m}\mu\text{M}$ of FAD in a final volume of 1.0 ml. was incubated for 25 to 60 minutes at 37°. (DPN and fluoride were added to inhibit pyrophosphatase activities as described below.) The fluorescence of an aliquot was then determined after dilution with water to 10 ml., and a blank, obtained by incubating enzyme and DPN in the absence of FAD, was subtracted from the read-

ing. In all experiments, the values obtained were compared with a control in which enzyme and DPN were absent (no FAD splitting) and with a standard FMN solution prepared from FAD with nucleotide pyrophosphatase (100 per cent FAD splitting).

Protein concentration of enzyme solutions was determined by the nephelometric method of Bücher (8) with the Beckman spectrophotometer at 340 $m\mu$.

Results

Purification of Enzyme

Procedure—100 gm. of beer yeast,² dried as previously described (4), were autolyzed with 300 ml. of 0.1 M sodium bicarbonate (saturated with a mixture of 95 per cent N_2 and 5 per cent CO_2) for 24 hours at 23°. All subsequent operations, including storage of solutions, were carried out at 3°, unless otherwise specified. The mixture was centrifuged, and the supernatant (autolysate, Table I) diluted with water to 324 ml. The precipitate obtained by adding 108 gm. of ammonium sulfate was centrifuged, dissolved in 60 ml. of water, and dialyzed against running, demineralized water for 1 hour. The dialyzed solution (Fraction I) was diluted to 75 ml. with water and mixed with 75 ml. of 0.1 M sodium acetate buffer (pH 5.0). After 5 minutes, 12 ml. of 95 per cent ethanol were added dropwise with mechanical stirring at 0° to -1° and the precipitate collected by centrifugation was discarded. To the supernatant were added another 23 ml. of 95 per cent ethanol at -2°. The precipitate was centrifuged and dissolved in 45 ml. of water and sufficient 0.1 N NaOH to give a nearly neutral solution which was then adjusted to pH 5.85 by cautious addition of 0.02 N acetic acid (Fraction II). To the solution were added 13.8 ml. of aluminum hydroxide gel C γ (9) (dry weight 15.5 gm. per liter). The suspension was centrifuged after 10 minutes, and the adsorbate washed with 13 ml. of 0.02 M sodium acetate buffer (pH 6.0) and eluted with three 14 ml. portions of 0.02 M phosphate buffer (pH 7.7). The combined eluates (Fraction III) were diluted to 48 ml. with water, and 1.0 ml. of 1.0 N acetic acid was added with mechanical stirring at 0°, followed by 6.9 ml. of 95 per cent ethanol at -1° to -2°. The precipitate was centrifuged and dissolved with 20 ml. of water and sufficient 0.1 N NaOH to give a nearly neutral solution which was then adjusted to pH 6.0 with 0.02 N acetic acid (Fraction IV). It was then treated with 21.4 ml. of calcium phosphate gel (10) (dry weight 8.2 gm. per liter) and centrifuged after 10 minutes. The adsorbate was washed with 17 ml. of 0.02 M sodium acetate buffer (pH 6.0) and eluted with four 4.3 ml. portions of 0.01 M phosphate

² Kindly furnished by Mr. F. R. Omlor of the Christian Heurich Brewing Company, Washington, D. C.

buffer (pH 7.7). The combined eluates (Fraction V) were clear and colorless. The protein content varied between 1.3 and 1.9 mg. per ml. This fraction represents an over-all yield of 45 per cent and a 91-fold purification as compared to the autolysate.

Stability and pH Effect—The enzyme (Fraction V), at 3°, lost 20 to 30 per cent of its activity in 4 days and 58 per cent in 11 days. At pH 6.0 the enzyme was 10 per cent, and at pH 8.4, 64 per cent as active as at pH 7.5.

Nucleotide Pyrophosphatase and Inorganic Pyrophosphatase in Purified Enzyme—Since the most purified enzyme fractions contained high concentrations of these activities, specific inhibitors were employed to avoid their interference in the study of reaction (1).

TABLE II

FAD Splitting by Nucleotide Pyrophosphatase in Purified Enzyme Preparation

The incubation mixtures (1.0 ml.) were as follows (in addition to the components listed): Experiment 1, 0.14 mg. of Fraction V, 2 μM of phosphate buffer (pH 7.7), 4.5 μM of MgCl_2 , 60 minutes; Experiment 2, 1.0 mg. of Fraction V, 45 μM of phosphate buffer (pH 7.5), 11.3 μM of MgCl_2 , 45 minutes, 37°.

Experiment No.	FAD	DPN	ATP	FAD split
	<i>mμM</i>	<i>mμM</i>	<i>mμM</i>	<i>mμM</i>
1	1.6			1.4
	1.6	2000		0.0
	1.6		1000	0.0
2	5.7			3.3
	5.7	5000		0.0

The presence of nucleotide pyrophosphatase was indicated by the hydrolysis of both DPN and FAD. With the assay method previously described (6), it was found that 20 $\text{m}\mu\text{M}$ of DPN were split by 0.68 mg. of Fraction V in 3 hours. Also, as shown in Table II, FAD was split in the absence of PP, and this FAD cleavage was completely inhibited by either DPN or ATP. FAD, DPN, and ATP are all substrates of nucleotide pyrophosphatase (6), and the presence of a large excess of one of these nucleotides effectively inhibits the splitting of limited concentrations of the others. Accordingly, studies of FAD breakdown by PP and studies of FAD synthesis with limited ATP were carried out in the presence of a large excess of DPN.

The presence of inorganic pyrophosphatase was demonstrated by incubating 4 μM of sodium pyrophosphate with 1.5 μM of MgCl_2 and 0.28 mg. of Fraction V (dialyzed for 4 hours against running water) in 1.0 ml. of 0.05 M glycylglycine buffer (pH 7.4) for 10 minutes at 38°. An analysis

for orthophosphate (11) showed that $0.165\ \mu\text{M}$ of PP had been split. In the presence of $0.05\ \text{M}$ sodium fluoride no cleavage could be detected. Therefore fluoride was added in studies of the breakdown of FAD by limited amounts of PP in order to prevent the removal of PP by inorganic pyrophosphatase action.

FAD Synthesis

Participation of FMN—In the absence of FMN, no FAD formation was detected in the assay. With ATP present in excess, the conversion of FMN to FAD was nearly quantitative. In an experiment with $0.17\ \text{mg.}$ of Fraction V, $1.5\ \mu\text{M}$ of MgCl_2 , $14\ \mu\text{M}$ of phosphate buffer (pH 7.5), $1.44\ \mu\text{M}$ of ATP, and $1\ \mu\text{M}$ of FMN (in $1.0\ \text{ml.}$ at 37°), the amounts of FAD

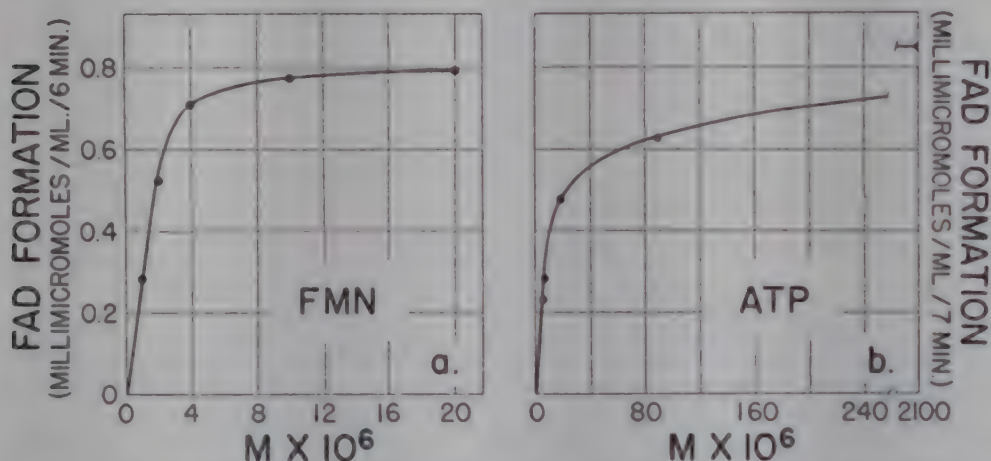


FIG. 1. Rate of FAD formation as a function of (a) FMN and (b) ATP concentration. The incubations were as follows: (a) $0.052\ \text{mg.}$ of Fraction V, $1.5\ \mu\text{M}$ of MgCl_2 , $25\ \mu\text{M}$ of phosphate buffer (pH 7.5), $1.44\ \mu\text{M}$ of ATP, and FMN as indicated, in a volume of $1.0\ \text{ml.}$, 6 minutes at 37° ; (b) $0.15\ \text{mg.}$ of Fraction V, $7.5\ \mu\text{M}$ of MgCl_2 , $10\ \mu\text{M}$ of phosphate buffer (pH 7.5), $3\ \mu\text{M}$ of DPN, $8\ \text{m}\mu\text{M}$ of FMN, and ATP as indicated, in a volume of $1.0\ \text{ml.}$, 7 minutes at 37° .

formed after periods of 10, 20, and 30 minutes were 0.85 , 0.93 , and $0.87\ \text{m}\mu\text{M}$, respectively. The rate of FAD formation as a function of FMN concentration is shown in Fig. 1, a. The dissociation constant (K_s) of the enzyme-substrate complex (12), derived from the substrate concentration at half maximal rate, is 1.4×10^{-6} mole per liter.

FAD Synthesis from Riboflavin—When FMN was replaced by comparable amounts of riboflavin, there was no measurable production of FAD. However, with increased amounts of enzyme and riboflavin a small but definite synthesis of FAD was observed. Thus with $0.38\ \text{mg.}$ of Fraction V, $7.5\ \mu\text{M}$ of MgCl_2 , $25\ \mu\text{M}$ of phosphate buffer (pH 7.5), $2\ \mu\text{M}$ of ATP, and $44\ \text{m}\mu\text{M}$ of riboflavin (U. S. P., Merck) in a final volume of $1.0\ \text{ml.}$, the amounts of FAD formed after 15 and 38 minutes at 37° were 0.15 and

0.25 μM respectively. This corresponds to a riboflavin conversion of 0.34 and 0.57 per cent.

Participation of ATP—In the absence of ATP, no FAD synthesis occurred. With FMN present in excess, conversion of ATP to FAD reached 60 per cent (Table III). The relatively high dissociation constant of the ATP-enzyme complex and the instability of the purified enzyme prevented

TABLE III
Conversion of ATP to FAD

Enzyme (Fraction V), 1.5 μM of MgCl_2 , 25 μM of phosphate buffer (pH 7.5), 3 μM of DPN, 1.44 $\text{m}\mu\text{M}$ of ATP, and 20 $\text{m}\mu\text{M}$ of FMN in a volume of 1.0 ml. were incubated for 20 minutes at 37°.

Fraction V	FAD formed	ATP converted
<i>mg. protein</i>	<i>mμM</i>	<i>per cent</i>
0.17	0.480	33
0.34	0.644	45
0.68	0.868	60

TABLE IV
Specificity of ATP in FAD Synthesis

Experiment 1, 0.19 mg. of Fraction V, 7.5 μM of MgCl_2 , 25 μM of phosphate buffer (pH 7.5), 3 μM of DPN, 4 $\text{m}\mu\text{M}$ of FMN, and adenine nucleotides as indicated, in 1.0 ml., 15 minutes at 37°. Experiment 2, 0.38 mg. of Fraction V, 7.5 μM of MgCl_2 , 25 μM of phosphate buffer (pH 7.5), 3 μM of DPN, 8 $\text{m}\mu\text{M}$ of FMN and adenine nucleotides as indicated, in 1.0 ml., 13 minutes at 37°.

Experiment No.	Nucleotide	Concentration	FAD formed
		<i>mμM per ml.</i>	<i>mμM per ml.</i>
1	Adenosine-5-phosphate	2000	0.00
	ADP*	2000	0.58
	ATP	2000	2.03
2	ADP*	4	0.03
	"	6	0.04
	"	2000	0.92
	ATP	6	0.70

* Contained 2.3 per cent ATP (see the text).

a quantitative conversion of ATP. The dependence of rate of FAD synthesis on ATP concentration is shown in Fig. 1, *b*; the K_s is 1.2×10^{-6} mole per liter.

Replacement of ATP by adenosine-5-phosphate resulted in no FAD synthesis (Table IV). While ADP in large amounts partially replaced ATP, it was only slightly effective at lower concentrations. An analysis

for ATP (4) of the ADP preparation used indicated a content of 2.3 per cent. From this amount of ATP contamination and the dissociation constant of the ATP-enzyme complex, it was calculated that the amount of

TABLE V
Mg⁺⁺ Requirement for FAD Synthesis

The mixtures contained 0.17 mg. of Fraction V, MgCl₂ as indicated, 12.5 μ M of phosphate buffer (pH 7.5), 1.44 μ M of ATP, and 10 m μ M of FMN in a volume of 1.0 ml. Incubation for 6 minutes at 37°. The FAD synthesis is expressed as millimicromoles per ml. per 6 minutes.

Mg ⁺⁺	FAD synthesis	Mg ⁺⁺	FAD synthesis
<i>M</i> $\times 10^3$		<i>M</i> $\times 10^3$	
0.0	0.05	0.45	0.85
0.038	0.11	0.75	1.35
0.075	0.17	1.50	1.73
0.18	0.34	3.00	1.13
0.30	0.70	7.50	0.95

TABLE VI
FAD Breakdown

The constituents were added in the following order: enzyme (Fraction V), phosphate buffer (pH 7.5) (3.5 μ M), MgCl₂, DPN (1.4 μ M), NaF (30 μ M), sodium pyrophosphate (pH 7.4), and FAD. Final volume 1.0 ml.; 37°. The amounts of enzyme were as follows: Experiment 1, 0.47 mg.; Experiment 2, 0.38 mg.; Experiment 3, 0.61 mg. (lower activity than in Experiments 1 and 2 because of aging).

Experiment No.	Time	FAD	PP	MgCl ₂	FAD split
	<i>min.</i>	<i>mμM</i>	<i>mμM</i>	<i>mμM</i>	<i>per cent</i>
1	40	5.3	0	1000	0.0
	40	5.3	35	1000	49.5
	40	5.3	350	1000	88.2
	40	5.3	350	0	4.7
2	55	15.7	0	830	0.0
	25	15.7	28	830	7.4
	55	15.7	28	830	14.2
	25	15.7	280	830	13.2
	55	15.7	280	830	26.6
	25	15.7	280	830	13.2
3	30	5.0	340	1500	43.0
	30	5.0	0*	1500	2.7

* 340 m μ M of sodium metaphosphate (4) were present.

FAD formation at both the lower and higher ADP levels could easily result from the ATP present in this ADP sample.

Mg⁺⁺ Requirement—The effect of Mg⁺⁺ on FAD synthesis is shown in

Table V. The optimal concentration appears to be about 1.5×10^{-3} M, with a diminished effect observed at higher levels. High concentrations (7.5×10^{-3} M) were used in the enzyme assay and other experiments before this "inhibitory" effect was discovered.

FAD Breakdown

The splitting of FAD was measured fluorometrically and, for reasons previously discussed, DPN and fluoride were included in the incubation mixture. From the data of Table VI it may be noted that the conversion of FAD to FMN required the presence of PP and Mg^{++} and was increased nearly 2-fold by a 10-fold increase in the PP concentration. The amount of FAD split was roughly proportional to time; the rate was not affected by a 3-fold increase in the FAD concentration from 5.3×10^{-6} M to 15.7×10^{-6} M. When metaphosphate (4) replaced PP (Experiment 3), no significant breakdown of FAD was observed. Phosphate buffer (3.5×10^{-3} M) was present in all experiments and was used at this low level to minimize complex formation between magnesium, fluoride, and phosphate.

DISCUSSION

The specific requirement for ATP and FMN in FAD synthesis and for PP in FAD breakdown provides strong evidence that the reaction proceeds as formulated in equation (1). The small amount of FAD synthesis from riboflavin and ATP very likely involved a preliminary phosphorylation of riboflavin to yield FMN by another enzyme present in the preparation. Unfortunately, several factors made it difficult to carry out balance and equilibrium experiments comparable to those performed in studies of DPN synthesis (4). While the sensitivity of methods for analysis of FMN and FAD made it possible to work with millimicromolar amounts of these scarce materials, analyses for ATP and PP could not be carried out at this level. Also the low specific activity of the purified enzyme preparation, only 0.02 per cent as active as the purified DPN-synthesizing enzyme from yeast (4), and its instability made large scale and prolonged incubations impractical. Finally, the necessity for fluoride and Mg^{++} in large amounts relative to PP probably resulted in complex formation to an extent that could not be readily evaluated.

The FAD-synthesizing activity in the yeast autolysates was only about 1 per cent of that reported for DPN-synthesizing activity (4), but it is important to recall that the concentration of FAD in fresh yeast (5) is also very much lower than that of DPN (13). Of interest in this connection are the relatively low dissociation constants of the complexes formed by FMN and ATP with the FAD-synthesizing enzyme (1.4×10^{-6} and 1.2×10^{-5} mole per liter, respectively) as compared with those formed by

NMN and ATP with the DPN-synthesizing enzyme (1.5×10^{-4} and 4.6×10^{-4} mole per liter, respectively) (4).

SUMMARY

An enzyme has been partially purified from brewers' yeast which catalyzes the reversible reaction between riboflavin phosphate and adenosine triphosphate (ATP) on the one hand and flavin-adenine dinucleotide (FAD) and inorganic pyrophosphate on the other in the presence of magnesium ions.

ATP could not be replaced by adenosine diphosphate or adenosine-5-phosphate, and in the reverse reaction, inorganic pyrophosphate could not be replaced by ortho- or metaphosphate. With riboflavin and ATP a slight amount of FAD formation was observed.

BIBLIOGRAPHY

1. Klein, J. R., and Kohn, H. I., *J. Biol. Chem.*, **136**, 177 (1940).
2. Trufanov, A. V., *Biokhimiya*, **6**, 301 (1941); **7**, 188 (1942); *Chem. Abstr.*, **35**, 7499 (1941); **38**, 131 (1944).
3. Kornberg, A., *J. Biol. Chem.*, **176**, 1475 (1948).
4. Kornberg, A., *J. Biol. Chem.*, **182**, 779 (1950).
5. Warburg, O., and Christian, W., *Biochem. Z.*, **298**, 150 (1938).
6. Kornberg, A., and Pricer, W. E., Jr., *J. Biol. Chem.*, **182**, 763 (1950).
7. Burch, H. B., Bessey, O. A., and Lowry, O. H., *J. Biol. Chem.*, **175**, 457 (1948).
8. Bücher, T., *Biochim. et biophys. acta*, **1**, 292 (1947).
9. Willstätter, R., and Kraut, H., *Ber. chem. Ges.*, **56**, 1117 (1923).
10. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **124**, 397 (1938).
11. Lowry, O. H., and Lopez, J. A., *J. Biol. Chem.*, **162**, 421 (1946).
12. Michaelis, L., and Menten, M. L., *Biochem. Z.*, **49**, 333 (1913).
13. Schlenk, F., in A symposium on respiratory enzymes, Madison (1942).

ENZYMATIC SYNTHESIS OF TRIPHOSPHOPYRIDINE NUCLEOTIDE

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Von Euler and Vestin (1) demonstrated the synthesis of triphosphopyridine nucleotide (TPN) when diphosphopyridine nucleotide (DPN) was incubated with adenosine triphosphate (ATP) in the presence of crude yeast maceration juice. In subsequent reports (2) from the Stockholm laboratory, several mechanisms for this reaction were considered but none was established. With crude pigeon liver fractions, Mehler *et al.* (3) observed the formation of TPN when DPN and ATP were incubated together; no synthesis was observed in the absence of either DPN or ATP. While the findings with the yeast and liver preparations were compatible with a direct phosphorylation of DPN by ATP, it was difficult to reconcile the observations of Altman and Evans (4) with this mechanism. These investigators found that TPN synthesis by an aqueous extract of pigeon liver acetone powder took place without added DPN. Also, adenylic acid replaced ATP, and nicotinamide originally present in the aqueous extract was completely converted to TPN. Most recently, studies on the enzymatic synthesis of DPN and flavin-adenine dinucleotide (FAD) have suggested another possible mechanism (5, 6). The demonstrated condensation of nicotinamide mononucleotide (NMN) and flavin mononucleotide with ATP to form DPN and FAD, respectively, made it desirable to ascertain whether an analogous mechanism obtained for TPN synthesis.

An enzyme has now been partially purified from autolysates of ale yeast which catalyzes the synthesis of TPN by a direct phosphorylation of DPN by ATP:



In this report are presented the evidence for this reaction and the purification of the enzyme which mediates it.

Methods

Materials—DPN, reduced DPN (DPNH₂), TPN, ATP, ADP, adenosine-5-phosphate, and NMN were prepared as previously described (5, 7). The sample of ADP contained 2.3 per cent ATP, as determined by the

reaction of glucose-6-phosphate (formed by the hexokinase system) with TPN in the *Zwischenferment* system.

Zwischenferment (Glucose-6-phosphate Dehydrogenase)—Crude preparations (8) contained impurities which interfered in the determination of ATP (5), and an attempt to prepare the purified enzyme (9) from our yeast was unsuccessful. With a new method of purification a product satisfactory for purposes of ATP assay was obtained. The purified preparation was only about one-third as pure as the best preparation of Negelein and Gerischer, but was 10 times as pure as crude *Zwischenferment* (8).

Purification was based on the rate of density increase at 340 m μ caused by the reduction of TPN in the test system, 0.5 cc. of glycylglycine buffer (0.25 M, pH 7.4), 0.1 cc. of MgCl₂ (0.3 M), 0.1 cc. of TPN (0.0015 M), 0.1 cc. of glucose-6-phosphate¹ (0.025 M), 0.005 to 0.025 unit of enzyme activity, and water to 3.0 cc. Readings in an absorption cell (1 cm. length) were taken every half minute for 2 minutes. A unit of *Zwischenferment* activity was defined as the amount causing the reduction of 1 μ M (micro-mole) of TPN (2.07 density units) per minute and specific activity as units per mg. of protein.

The procedure was as follows: 50 gm. of dried brewers' yeast² were suspended in 150 cc. of 0.1 M sodium bicarbonate (saturated with 5 per cent CO₂) and kept at 40° for 5 hours. Subsequent operations were carried out at 2°. The clear autolysate (89 cc., 410 units, 0.10 unit per mg.) obtained by centrifugation could be stored overnight without loss. Sodium bicarbonate (267 cc., 0.1 M, saturated with 5 per cent CO₂ at 23°) and then ammonium sulfate (125 gm.) were added to the autolysate. The mixture was centrifuged, the precipitate discarded, and ammonium sulfate (26.7 gm.) was added to the supernatant. The precipitate was collected by centrifugation and dissolved in water (560 cc., 260 units, 0.23 unit per mg.). The enzyme was adsorbed on 139 cc. (1.10 gm. dry weight) of calcium phosphate gel (10) and eluted with 223 cc. of cold 0.5 M phosphate buffer, pH 7.7. Ammonium sulfate (75 gm.) was added to the eluate and the precipitate separated by centrifugation was discarded. A second precipitate was obtained by the addition of 13.3 gm. of ammonium sulfate to the supernatant, collected by centrifugation, and dissolved in water (44 cc., 141 units, 1.6 units per mg.). To this ammonium sulfate fraction were added 220 cc. of acetate buffer (0.1 M, pH 4.4). After 10 minutes at 0°, the precipitate was collected by centrifugation, dissolved in 6.0 cc. of glycylglycine buffer (0.25 M, pH 7.4), frozen, and dried *in vacuo*. The white powder (230 mg., 125 units, 2.0 units per mg.) is stable for months at 2°.

In the presence of glycylglycine buffer (0.04 M, pH 7.4), the addition

¹ Kindly furnished by Dr. B. L. Horecker.

² Kindly furnished by Anheuser-Busch, Inc., St. Louis.

of Mg^{++} (0.01 M) produced a 3- to 4-fold increase in *Zwischenferment* activity with crude as well as with the purified preparations. When phosphate buffer (0.04 M, pH 7.4) was used, the activating effect of Mg^{++} was slight and the rates approached those obtained with glycylglycine and Mg^{++} . These findings and the ability of glycylglycine to form complexes with Mg^{++3} suggest that *Zwischenferment* may prove to be a metal-requiring dehydrogenase.

Determinations—DPN was determined with the model DU Beckman spectrophotometer by reduction with alcohol and crystalline alcohol dehydrogenase (5).

$DPNH_2$ was determined spectrophotometrically by oxidation with pyruvate and lactic dehydrogenase (11).

ATP estimations were based on TPN reduction by the combined action of the hexokinase and *Zwischenferment* systems as described (5) with one modification. The hexokinase content of the *Zwischenferment* preparation was high enough to eliminate the requirement for the separate addition of hexokinase. Analytical difficulties previously encountered (5) with crude *Zwischenferment* were avoided by the use of the more purified preparation.

TPN was determined spectrophotometrically by reduction with isocitric dehydrogenase and *dl*-isocitric acid (12),⁴ a reaction of known high specificity for TPN (3). In some instances, determinations were also made by reduction with *Zwischenferment* and glucose-6-phosphate. Identical results were obtained with both methods.

Phosphate was determined by the method of Lowry and Lopez (13) and protein nephelometrically according to Bücher (14).

Enzyme Assay (Rate of TPN Synthesis)—The components of the test were as follows: 0.05 cc. of DPN (0.05 M), 0.05 cc. of ATP (0.035 M), 0.1 cc. of $MgCl_2$ (0.15 M), 0.1 cc. of phosphate buffer (0.5 M, pH 7.0), 0.2 to 1.0 unit of enzyme, and water to 1.0 cc. After incubation for 20 minutes at 38°, the reaction was stopped by the addition of 1.0 cc. of 10 per cent trichloroacetic acid. An aliquot (1.0 cc.) of the neutralized, protein-free supernatant (2.5 cc.) was analyzed for TPN. With the purified preparations, the reaction was stopped by immersion in boiling water for 30 seconds and the coagulated protein was removed by centrifugation. No significant TPN synthesis was detected with this assay method when applied to crude autolysates, perhaps as a result of some inhibitory factor in the autolysates. A unit of enzyme activity was defined as the amount causing the synthesis of 1 μM of TPN per hour and specific activity as units per mg. of protein.

³ Kornberg, A., Ochoa, S., and Mehler, A. H., unpublished observations.

⁴ Kindly furnished by Dr. S. Ochoa.

Results

Purification of Enzyme—A preliminary survey was made of the potencies of several brewers' yeasts under various conditions of autolysis. The following procedure was adopted. 50 gm. of dried (5) top ale yeast⁵ were suspended in 150 cc. of sodium bicarbonate (0.1 M, saturated with 5 per cent CO₂) and kept at 23° for 40 hours. Subsequent operations were carried out at 2°. The mixture was centrifuged and to the supernatant fluid (105 cc.) were added an equal volume of cold water and then 70 gm. of ammonium sulfate. The precipitate was collected by centrifugation and dissolved in water (ammonium sulfate, Fraction A, Table I). The enzyme was adsorbed on 46 cc. (360 mg. dry weight) of calcium phosphate gel, the gel washed with 125 cc. of cold water, and then the enzyme eluted with 185 cc. of cold phosphate buffer (0.5 M, pH 7.7). To this

TABLE I
Purification of TPN-Synthesizing Enzyme

Step	Volume	Total activity	Over-all yield	Specific activity
	cc.	units	per cent	units per mg. protein
1. Ammonium sulfate, Fraction A.....	310	76		0.11
2. Calcium phosphate gel eluate.....	185	57	75	0.66
3. Ammonium sulfate, Fraction B	62	57	75	0.90
4. Ppt. at pH 4.4.....	100	51	67	1.01
5. Aluminum hydroxide gel eluate.....	65	28	37	1.95
6. Ammonium sulfate, Fraction C	5	22	29	2.70

eluate (Table I) were added 50 gm. of ammonium sulfate, and the precipitate was collected by centrifugation and dissolved in water (ammonium sulfate, Fraction B). Acetate buffer (250 cc., 0.1 M, pH 4.4) was added. After 10 minutes, the precipitate was centrifuged off and dissolved in glycylglycine buffer (0.1 M, pH 7.4) (precipitate at pH 4.4, Table I). At this stage, the enzyme could be kept at -15° for at least several days without loss in activity. The solution was dialyzed for 45 minutes against running distilled water, and treated first with an equal volume of cold acetate buffer (0.1 M, pH 6.0) and then with 22 cc. (341 mg. dry weight) of aluminum hydroxide gel C γ (15). The gel was washed with two 43 cc. portions of cold phosphate buffer (0.1 M, pH 7.4) and the enzyme eluted with two 33 cc. portions of cold phosphate buffer (0.5 M, pH 7.7). To the eluate (Table I) were added 20 gm. of ammonium sulfate.

⁵ Kindly furnished by Mr. F. X. Schneider of the Gunther Brewing Company, Baltimore.

The precipitate was washed in the centrifuge with cold 0.50 saturated ammonium sulfate and dissolved in glycylglycine buffer (0.1 M, pH 7.4) (ammonium sulfate, Fraction C). This purified preparation lost 50 per cent of its activity after 48 hours at 0°, but was stable for weeks when stored at -15°.

Balance Studies of TPN Synthesis—In the absence of either DPN or ATP, there was no detectable TPN synthesis. As shown in Table II, a maximal rate of TPN synthesis required approximately 2×10^{-3} M ATP

TABLE II

Balance Studies of TPN Synthesis

The incubation mixtures (1.0 cc.) contained 15 μ M of $MgCl_2$, 50 μ M of glycylglycine buffer (pH 7.4), 0.30 mg. of purified enzyme (ammonium sulfate, Fraction C), and the amounts of DPN and ATP indicated. 30 minutes at 38°. The values are expressed in micromoles.

Experiment No.	DPN added	ATP added	- Δ DPN	- Δ ATP	+ Δ TPN
1	1.08*	0.00	0.00		0.00
	1.08	0.00	0.01		0.00
	1.08	0.72	0.13		0.12
	1.08	1.44	0.20		0.16
	1.08	2.88	0.20		0.17
	1.08	7.20	0.19		0.17
2	0.00*	0.57		0.00	0.00
	0.00	0.57		0.12	0.00
	1.08	0.57	0.13	0.13†	0.12
	2.16	0.57		0.14†	0.15
	4.32	0.57		0.16†	0.18
	13.0	0.57		0.18†	0.21

* Enzyme omitted.

† Corrected for ATP disappearance due to adenosine triphosphatase action (see the text).

and even higher concentrations of DPN. The amounts of TPN synthesized were approximately equivalent to the amounts of DPN and ATP removed. Determinations of ATP removal required a correction for ATPase activity which was present in the purified preparations. Thus, in Experiment 2, 0.12 μ M of ATP disappeared in the absence of added DPN and 0.13 μ M of inorganic orthophosphate was liberated. The presence of varying amounts of DPN (as in Experiment 2, Table II) did not affect the amount of orthophosphate liberated from ATP.

Failure of NMN to Replace DPN—With NMN in place of DPN a small amount of TPN synthesis took place. However, as shown in Table III, this amount of reaction can be readily accounted for by a preliminary

synthesis of DPN due to the presence of the enzyme which catalyzes DPN production from NMN and ATP (5). Consistent with this explanation is the fact that inorganic pyrophosphate in high concentration inhibited DPN synthesis 87 per cent (as would be anticipated from equilibrium considerations) and completely prevented TPN synthesis from NMN, but inhibited TPN synthesis from DPN by only 41 per cent. While

TABLE III

Failure of NMN to Replace DPN in TPN Synthesis

The incubation mixtures (1.0 cc.) contained 3 μM of MgCl_2 , 50 μM of glycylglycine buffer (pH 7.4), 0.30 mg. of purified enzyme (ammonium sulfate, Fraction C), and the amounts of NMN, DPN, and inorganic pyrophosphate as indicated. 30 minutes at 38°. The values are expressed in micromoles.

NMN added	DPN added	Inorganic pyrophosphate added	DPN synthesis	TPN synthesis
5	0	0	0.253	0.024
5	0	20	0.033	0.000
0	5	0		0.357
0	5	20		0.209

TABLE IV

Failure of ADP to Replace ATP in TPN Synthesis

The incubation mixtures (1.0 cc.) contained 15 μM of MgCl_2 , 50 μM of glycylglycine buffer (pH 7.4), 2 μM of DPN, 0.30 mg. of purified enzyme (ammonium sulfate, Fraction C), and the amounts of ADP and ATP indicated. 30 minutes at 38°. The values are expressed in micromoles.

ATP added	ADP* added	TPN synthesis
0.37	0.00	0.113
0.74	0.00	0.169
0.00	0.35	0.009
0.00	0.70	0.008

* Contained 2.3 per cent ATP (see the text).

it is clear that NMN did not participate in TPN synthesis with the purified enzyme, the possibility remains that it might be involved in some alternative mechanism. Thus far no evidence has been obtained in studies with crude yeast fractions to support this possibility.

Failure of ADP to Replace ATP—When ADP replaced ATP, only traces of TPN were produced (Table IV). The ATP contamination of this ADP sample (2.3 per cent) was sufficient to account for this effect.

Effect of Mg^{++} , Mn^{++} , and pH—The stimulation of TPN synthesis by Mg^{++} and Mn^{++} is shown in Fig. 1. The optimal concentration for Mg^{++}

was from 5 to 10×10^{-3} mole per liter and for Mn^{++} 1×10^{-3} ; higher concentrations were less effective. The optimal pH for TPN synthesis appeared to be near 8. With 0.05 M phosphate at pH 6.0, 6.5, 7.0, 7.5, and 8.0, the respective rates were 1.8, 4.3, 6.4, 7.7, and 8.0 units per cc.

Conversion of $DPNH_2$ to $TPNH_2$ —The phosphorylation of $DPNH_2$ by ATP to form $TPNH_2$ was determined in two ways. One method was the reduction of cytochrome *c* by cytochrome reductase (specific for $TPNH_2$) which was isolated by Horecker (16). With excess cytochrome *c* and 10

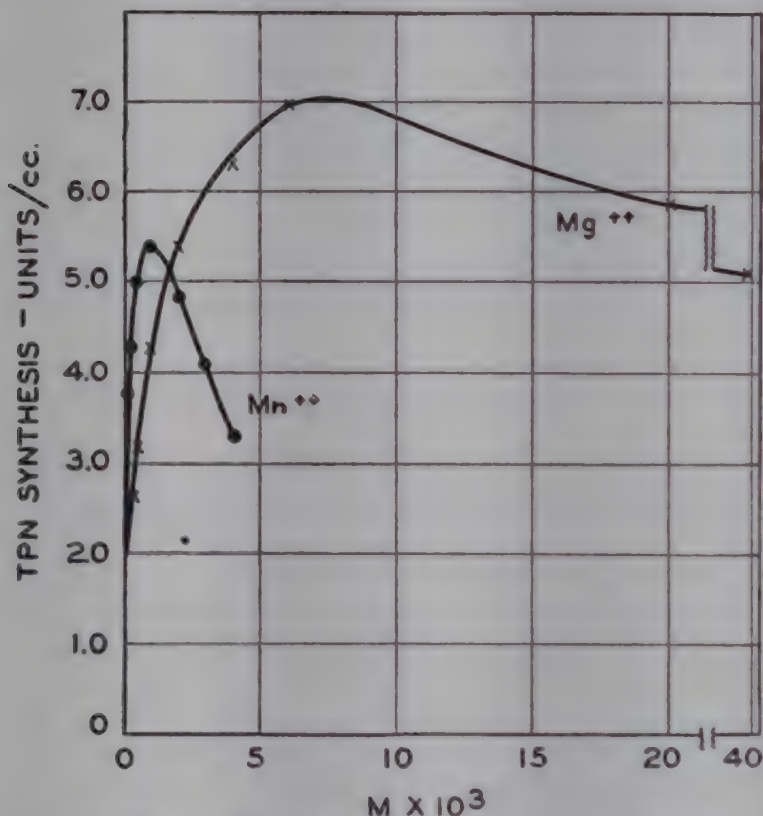


FIG. 1. Effects of Mg^{++} and Mn^{++} on the TPN-synthesizing enzyme. 0.25 mg. of purified enzyme was present; for other details, see "Methods."

γ of 15 per cent pure cytochrome reductase,¹ the oxidation of $TPNH_2$ was complete within 2 minutes as measured by the increase in density at $550\text{ m}\mu$. The concentration of reduced cytochrome *c* was determined by dividing the increase in density by $1.96 \times 10^7\text{ cm}^2\text{ mole}^{-1}$, a value which represents the difference between the extinction coefficients of reduced and oxidized cytochrome *c*. The second method involved the oxidation of $TPNH_2$ by excess pyruvate and lactic dehydrogenase (3). By proper adjustment of the amount of lactic dehydrogenase used, the $DPNH_2$ and $TPNH_2$ content of a mixture of the two nucleotides could be determined. With as little as $0.5\text{ }\gamma$ of a purified lactic dehydrogenase preparation,⁶ 0.1

⁶ Obtained by repeated ammonium sulfate fractionation of the supernatant fluid after crystallization of aldolase (17).

μM of DPNH_2 in a 3 cc. volume was completely oxidized within 2 minutes. On the other hand, there was no oxidation of $0.1 \mu\text{M}$ of TPNH_2 in a 3 cc. volume in a 5 minute period with as much as 25 γ of the lactic dehydrogenase preparation. By increasing the lactic dehydrogenase addition to 1 mg., the TPNH_2 was oxidized, and 30 to 45 minutes were required to complete the oxidation.

The incubation mixture for TPNH_2 synthesis was as follows: $0.23 \mu\text{M}$ of DPNH_2 , $3.8 \mu\text{M}$ of ATP, $10 \mu\text{M}$ of MgCl_2 , $50 \mu\text{M}$ of phosphate buffer, pH 7.7, 0.5 mg. of purified yeast enzyme, and water to 1.0 cc. A control mixture lacked only the enzyme. After 30 minutes at 38° , the enzyme was inactivated by adding 0.25 cc. of 0.1 N NaOH and placing the mixture in boiling water for 1 minute. The mixture was cooled, neutralized,* and centrifuged clear. Aliquots of the control and experimental mixtures were analyzed for DPNH_2 and TPNH_2 with the following results:

+ Δ TPNH_2 (cytochrome <i>c</i> method)	= $0.35 \mu\text{M}$
+ Δ " (lactic dehydrogenase method)	= 0.36 "
- Δ DPNH_2	= 0.41 "

DISCUSSION

The stoichiometric balance between TPN synthesis and the disappearance of DPN and ATP, plus the failure of NMN and ADP to react, leave little reason to doubt the validity of reaction (1). The lack of an analytical method for the determination of ADP, as specific, sensitive, and accurate as those for the other components in the reaction, prevented the inclusion of ADP in these balance studies. Isolation of ADP and TPN was not attempted because of the poor conversion of DPN and ATP. This was due to the high dissociation constants of the DPN- and ATP-enzyme complexes and the relatively low activity and unstable nature of the purified enzyme preparation. The question whether reaction (1) provides the only mechanism for TPN synthesis cannot be answered, but it appears unnecessary on the basis of available data to postulate the existence of an additional mechanism.

SUMMARY

An enzyme has been partially purified from autolysates of ale yeast which catalyzes the synthesis of TPN by a direct phosphorylation of DPN by ATP in the presence of Mg^{++} or Mn^{++} . The same system converts DPNH_2 to TPNH_2 .

The help of Mr. W. E. Pricer, Jr., in part of this work is gratefully acknowledged.

BIBLIOGRAPHY

1. von Euler, H., and Vestin, R., *Ark. Kemi, Mineral. o. Geol.*, **12B**, No. 44 (1938).
2. von Euler, H., and Adler, E., *Z. physiol. Chem.*, **252**, 41 (1938). Adler, E., Elliott, S., and Elliott, L., *Enzymologia*, **8**, 80 (1940).
3. Mehler, A. H., Kornberg, A., Grisolia, S., and Ochoa, S., *J. Biol. Chem.*, **174**, 961 (1948).
4. Altman, K. I., and Evans, E. A., Jr., *J. Biol. Chem.*, **169**, 463 (1947).
5. Kornberg, A., *J. Biol. Chem.*, **182**, 779 (1950).
6. Schrecker, A. W., and Kornberg, A., *J. Biol. Chem.*, **182**, 795 (1950).
7. Kornberg, A., and Pricer, W. E., Jr., *J. Biol. Chem.*, **182**, 763 (1950).
8. Warburg, O., and Christian, W., *Biochem. Z.*, **254**, 438 (1932).
9. Negelein, E., and Gerischer, W., *Biochem. Z.*, **284**, 289 (1930).
10. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **124**, 397 (1938).
11. Kubowitz, F., and Ott, P., *Biochem. Z.*, **314**, 94 (1943).
12. Ochoa, S., *J. Biol. Chem.*, **174**, 133 (1948).
13. Lowry, O. H., and Lopez, J. A., *J. Biol. Chem.*, **162**, 421 (1946).
14. Bücher, T., *Biochim. et biophys. acta*, **1**, 292 (1947).
15. Willstätter, R., and Kraut, H., *Ber. chem. Ges.*, **56**, 1117 (1923).
16. Horecker, B. L., *Federation Proc.*, **8**, 207 (1949).
17. Taylor, J. F., Green, A. A., and Cori, G. T., *J. Biol. Chem.*, **173**, 591 (1948).

THE COMBINED MANOMETRIC DETERMINATION OF OXYGEN AND CARBON DIOXIDE IN BLOOD, IN THE PRESENCE OF LOW CONCENTRATIONS OF ETHYL ETHER

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The manometric technique of Van Slyke and Neill (1) for the combined determination of oxygen and carbon dioxide in blood cannot be applied without modifications if ethyl ether in anesthetic concentrations is present in the blood. Part of the ether introduced with the blood, or serum, into the chamber of the Van Slyke apparatus will vaporize and add to the volume of extracted gases. If the amount of ether in the gas phase remained unchanged throughout the procedure, an interference with the correct blood gas analysis might not be encountered. Owing to the high solubility coefficient of ether in water, a considerable portion of the extracted ether is reabsorbed when the sodium hydroxide solution is added for the absorption of carbon dioxide, and is absent in the final measurement (2). This reabsorption of ether, according to Austin (2), may result in values up to 15 per cent in excess of the true carbon dioxide values. Shaw and Downing (3) reported errors of a similar magnitude in oxygen determinations in the presence of ether.

Austin (2) introduced a modification of the Van Slyke-Neill procedure for the determination of carbon dioxide in serum in the presence of ether. Essentially, his modification consists of reextraction of the alkaline solution after absorption of carbon dioxide, and the use of an empirical correction factor to compensate for the increase of ether in the gas phase over the alkaline solution. Fuss and Derra (4) applied Austin's principle to the simultaneous determination of blood oxygen and carbon dioxide in the presence of ether. They provided no experimental data to prove the validity of their procedure. Shaw and Downing (3) were unable to obtain correct values for oxygen, using the modification of Fuss and Derra. Shaw and Downing (3) described another modification of the Van Slyke-Neill technique for the determination of blood oxygen in the presence of ether. Their method requires the use of a modified Hempel pipette to which the extracted gases are temporarily transferred, allowing for the cleaning of the chamber of the ether-containing blood-reagent mixture. The method produces satisfactory results, and with the use of a correction

factor is practically free from systematic errors; it is, however, time-consuming and cumbersome. Whether the technique of Shaw and Downing can also be used for the determination of carbon dioxide, or for the combined determination of oxygen and carbon dioxide, has not been reported.

A simple modification of the Van Slyke-Neill technique for the combined determination of oxygen and carbon dioxide in the presence of ether was needed in an investigation of the effects of controlled breathing anesthesia on the blood oxygen saturation and carbon dioxide tensions of anesthetized patients.¹ The concentration of ether in the blood of patients anesthetized when using the Crafoord (5) or Mautz (6) machines for controlled respiration was found never to exceed 0.7 mg. per ml. in any of the twenty-three patients studied. The modification which is described below is directly applicable to blood containing ether in concentrations not exceeding 0.7 mg. per ml.

Method

The reagents and apparatus are the same as the ones used in the unmodified procedure of Van Slyke and Neill (1, 7). The Van Slyke-Neill procedure is followed without changes up to the completion of the gas extraction and the taking of the first reading, p_1 . The sodium hydroxide is then added, the cup sealed, and the mercury in the chamber lowered to the 50 ml. mark, and the chamber shaken for 1 minute, timed with a stopwatch. A reading, $p_{2(I)}$, is then taken. Immediately thereafter the mercury is again lowered to the 50 ml. mark, the chamber is shaken for another minute, and another reading, $p_{2(II)}$, is taken. The value for p_2 corrected is obtained by using the formula, p_2 corrected equals $p_{2(I)} + (p_{2(I)} - p_{2(II)})$. The carbon dioxide content is calculated by means of the formula $(p_1 - p_{2\text{ cor.}} - c) \times f$. The values c and f are the same as those used in the ordinary Van Slyke-Neill procedure. The oxygen absorbent is then introduced and the solution shaken for 2 minutes under lowered pressure. Reading p_3 is taken. The oxygen content is calculated according to the formula $(p_{2\text{ cor.}} - p_3 - c) \times f$.

EXPERIMENTAL

On repeated extractions of the blood-reagent mixture after the addition of sodium hydroxide, successively lower manometric readings are obtained (Table I). Van Slyke and Stadie (8) noted, in connection with the alkaline ferri cyanide methods of oxygen determination used by earlier workers, that the alkaline reaction favored some oxidative processes by which part of the oxygen freed is slowly consumed. Hence, lower values for oxygen were obtained with these methods. Reducing substances in

¹ Stayman, J. W., Jr., Gibbon, J. H., Jr., and Allbritten, F. F., Jr., unpublished.

blood corpuscles (9) and plasma (10) were held responsible for the loss of oxygen in the alkaline ferricyanide methods. Yet this explanation could not account for lower oxygen capacity values of fresh hemoglobin solutions found with Haldane's alkaline ferricyanide method, as compared with values produced by the Van Slyke technique (11).

In the course of the present investigation it appeared that the oxygen consumption that apparently occurs when the alkaline blood-reagent mixture is shaken is at least to a large extent caused by the oxidation of caprylic alcohol and also saponin by ferricyanide. This oxidation apparently involves elementary oxygen. When a mixture of ferricyanide solution, sodium hydroxide, a few drops of caprylic alcohol, and air was admitted into the chamber of the Van Slyke apparatus, successively lower

TABLE I

Manometric Readings on Successive Extractions of Blood-Reagent Mixture before and after Addition of Sodium Hydroxide

	Reading No.	Time of shaking	Manometric reading
		min.	mm.
Before addition of NaOH	1	3	305.5
	2	3	305.5
After addition of 1 ml. 1 N NaOH	3	0	186.5
	4	3	184.0
	5	2	182.2
	6	3	180.0
	7	3	178.5
	8	3	177.8

manometric readings were obtained on repeated extractions (Table II). The mixture was then collected in a test-tube and a few drops of ferric chloride solution were added. The typical color of ferric ferrocyanide appeared, proof of the presence in solution of (potassium) ferrocyanide. Less, but still significant, oxygen consumption was observed when saponin, in amounts used for a single blood gas determination, was shaken with an alkaline ferricyanide solution. The addition of ferric chloride to the ferricyanide solution before shaking with caprylic alcohol, or saponin, did not produce the Prussian blue reaction. The oxygen consumption that occurred on shaking the Van Slyke reagents, including sodium hydroxide, with air, was comparable in magnitude to the oxygen consumption of the alkaline blood-reagent mixture. No oxygen consumption occurred when caprylic alcohol, or saponin, was shaken with ferricyanide in neutral or acid solution.

Austin, who introduced the principle of reextraction, used it only for

carbon dioxide determinations in which no potassium ferricyanide is employed. If reextraction is used for oxygen determinations, compensation has to be made for the oxygen consumption occurring during the period of shaking after the addition of sodium hydroxide. Such compensation has apparently been neglected by Fuss and Derra (4). The rate of oxidation can, for practical purposes, be assumed to remain constant within the two 1 minute shaking periods employed here. (A slight decrease in the oxidation rate actually occurs as the oxidizable substances are being used up.) Therefore, the difference in the readings, $p_2 \text{ (I)} - p_2 \text{ (II)}$, is added to $p_2 \text{ (I)}$ in order to obtain the hypothetical reading p_2 that would

TABLE II

Manometric Readings on Successive Extractions of Mixture of 5 Ml. of 1.5 Per Cent Potassium Ferricyanide Solution, 4 Drops of Caprylic Alcohol, and 0.5 Ml. of Air

The time of shaking was 3 minutes.

	Reading No.	Manometric reading
		<i>mm.</i>
Before addition of NaOH	1	327.8
	2	327.8
After addition of 1 ml. 1 N NaOH	3	324.0
	4	320.1
	5	317.1
	6	313.5

have been obtained without reextraction if no reabsorption of ether occurred. No correction for the p_3 reading taken after 2 minutes shaking need be applied.

Results

Twenty experiments, involving over 120 gas determinations, were performed to test the validity of the method. The procedure in these test experiments was as follows: 30 ml. of blood were aerated for 20 minutes. 10 ml. samples of the aerated blood were transferred, with minimum exposure, to each of two oiled 10 ml. Luer lock syringes, to one of which ethyl ether in amounts ranging between 3 and 30 mg. was added with a micro pipette. The syringes were immediately capped with soldered needle hubs and kept on ice until analysis. A drop of mercury was added to facilitate proper mixing of the blood. Oxygen and carbon dioxide determinations were performed, as a rule in triplicate, on the ether-free control samples by the Van Slyke-Neill technique, and on the ether-containing samples ("ether" samples) with the modification here described. At the conclusion of each experiment, the hematocrit of both samples was

determined as a check of the relative identity of the samples. Generally good agreement between the gas content of the control and "ether" samples was found, when the concentration of ether did not exceed 0.7 mg. per ml. The deviation from the control values in these instances was always

TABLE III

Comparison of Oxygen and Carbon Dioxide Values Found in Two Samples of Same Blood, One of Which Contained 0.6 Mg. of Ethyl Ether per Ml.

The determinations were performed in triplicate with the Van Slyke-Neill technique on a control sample, with the authors' modified technique on the "ether" sample.

Determination No.	Control sample					Ether sample				
	Manometric readings	($p_1 - p_2$)	($p_2 - p_3$)	CO ₂ content*	O ₂ content†	Manometric readings	($p_1 - p_2$ cor.)	(p_2 cor. - p_3)	CO ₂ content‡	O ₂ content§
	mm.	mm.	mm.	vol. per cent	vol. per cent	mm.	mm.	mm.	vol. per cent	vol. per cent
1	p_1 202.1 p_2 169.7 p_3 112.1	32.4	57.6	8.25	13.68	p_1 214.1 p_2 (I) 180.6 p_2 (II) 179.6 p_2 cor. 181.6 p_3 123.8	32.5	57.8	8.27	13.72
2	p_1 202.1 p_2 169.9 p_3 112.0	32.1	57.9	8.17	13.74	p_1 214.1 p_2 (I) 181.2 p_2 (II) 180.2 p_2 cor. 182.2 p_3 124.4	31.9	57.8	8.11	13.72
3	p_1 202.2 p_2 169.9 p_3 112.1	32.3	57.8	8.23	13.73	p_1 214.2 p_2 (I) 181.2 p_2 (II) 180.0 p_2 cor. 182.4 p_3 124.2	31.8	58.2	8.09	13.81
Mean of three determinations.....				8.22	13.72				8.16	13.75

* Calculated according to the formula, $(p_1 - p_2 - c) \times f$.

† Calculated according to the formula, $(p_2 - p_3 - c) \times f$.

‡ Calculated according to the formula, $(p_1 - p_2 \text{ cor.} - c) \times f$.

§ Calculated according to the formula, $(p_2 \text{ cor.} - p_3 - c) \times f$.

within the accepted limits of error of the Van Slyke-Neill technique; i.e., 0.2 volume per cent for oxygen and 0.5 volume per cent for carbon dioxide, but remained generally much lower. In ten experiments in which the ether concentration ranged between 0.3 and 0.7 mg. per ml. the mean difference between values found in the control and "ether" samples was -0.03 volume per cent for oxygen and -0.29 volume per cent for carbon dioxide. In almost all individual experiments the mean deviations be-

tween control and "ether" values were lower than the maximum deviations on triplicate determination of the same sample. Table III shows the result of a typical experiment. The error in carbon dioxide values, if calculated in per cent, would appear to be excessively high because the carbon dioxide content of the aerated blood was very low. Proof was obtained, however, that the magnitude of the error depends mainly on the concentration of ether and not on the concentration of blood gases. The direction of the error was toward lower rather than true values for both oxygen and carbon dioxide. This is due to the higher solubility of ether in acid solution than in alkaline, as explained by Austin (2). No correction was used, since the error with low anesthetic concentrations as encountered in our clinical investigation was negligible. With higher concentrations of ether, Austin's correction formula could probably be used, but was not tried here. The method has been successfully used in the previously mentioned clinical study on twenty-three patients.¹ Duplicate checks are easily obtained if proper care is exerted, consisting mainly in accurate timing of the extractions and smooth and even timed reducing of the gas volume after each extraction.

SUMMARY

A modification of the Van Slyke-Neill technique for the combined determination of blood oxygen and carbon dioxide, to be used in the presence of ethyl ether, is described. Data are presented to prove the validity of the method for low anesthetic concentrations of ether.

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BIBLIOGRAPHY

1. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, **61**, 523 (1924).
2. Austin, J. H., *J. Biol. Chem.*, **61**, 345 (1924).
3. Shaw, J. L., and Downing, V., *J. Biol. Chem.*, **109**, 405 (1935).
4. Fuss, H., and Derra, E., *Arch. exp. Path. u. Pharmacol.*, **156**, 64 (1930).
5. Crafoord, C., *J. Thoracic Surg.*, **9**, 237 (1939-40).
6. Mautz, F. R., *J. Thoracic Surg.*, **10**, 544 (1940-41).
7. Peters, J. P., and Van Slyke, D. D., *Quantitative clinical chemistry, Methods*, Baltimore (1932).
8. Van Slyke, D. D., and Stadie, W. C., *J. Biol. Chem.*, **49**, 1 (1921).
9. Morawitz, P., *Arch. exp. Path. u. Pharmacol.*, **60**, 298 (1909).
10. Parsons, T. R., and Parsons, W., *Biochem. J.*, **21**, 1194 (1927).
11. Abeloos, M., Barcroft, J., Cordero, N., Harrison, T. R., and Sendroy, J., *J. Physiol.*, **66**, 262 (1928).

A PROCEDURE FOR THE DETERMINATION OF PROTEOLYTIC ACTIVITY*

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The difficulties introduced by the desire to maintain a constant pH during an enzyme-catalyzed hydrolysis of peptide-like substrates and at the same time to determine the extent of hydrolysis by an acid-base titration have been pointed out (1), but to date no completely satisfactory solution of the problem has been given. With those enzymes whose pH optima lie in the region between pH 7.5 to 8.5, *e.g.* trypsin and chymotrypsin, the poor buffering capacity of phosphate in this region prompted us, as it has others (2-5), to consider the use of organic amines whose pK'_a values were near to or identical with the pH optimum of the enzyme being used. In the course of such studies it soon became evident that coincidental use of a suitable primary or secondary amine buffer system and a formol titration (1) would insure adequate buffering capacity with low buffer concentration during the hydrolysis and at the same time permit the final acid-base titration to be conducted under nearly ideal conditions. In this communication we shall limit the discussion to results obtained with chymotrypsin and specific acylated- α -amino acid amide substrates, since the application of the general method to other proteolytic enzymes and other types of substrates will be obvious.

Initially the system $\overset{+}{\text{NH}}_3\text{CH}_2\text{CH}_2\overset{+}{\text{NH}}_3-\overset{+}{\text{NH}}_3\text{CH}_2\text{CH}_2\text{NH}_2-\text{NH}_2\text{CH}_2\text{CH}_2-\text{NH}_2$ ($pK'_{a_1} = 10.0$; $pK'_{a_2} = 7.0$) (6) was employed as a buffer for chymotrypsin studies at pH 7.8¹ and, while superior to phosphate or veronal, was subsequently discarded in favor of the system $(\text{CH}_2\text{OH})_3\overset{+}{\text{CNH}}_3-(\text{CH}_2\text{OH})_3\text{CNH}_2$ ($pK'_a = 8.1$) which is not only an excellent buffer at pH 7.8 (7) but is also monovalent. While formaldehyde would be expected to react with either a primary or secondary amine, the titration curves given in Figs. 1 and 2 will serve to emphasize the point that the reaction is not necessarily quantitative or irreversible (1). Thus for any given case in which maximum accuracy is desired it is clear that, as for other formol titrations (1), the end-point of the titration must be determined experimentally and in the system containing buffer, substrate,

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† Contribution No. 1334.

¹ Unpublished experiments of R. V. MacAllister.

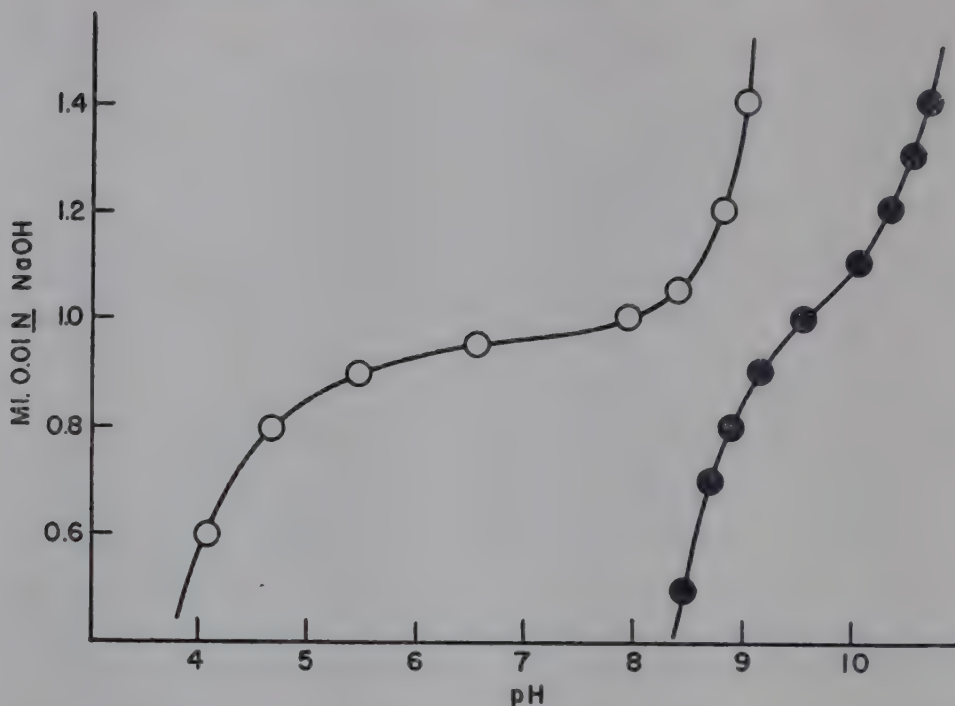


FIG. 1. Titration of tris(hydroxymethyl)aminomethane-hydrochloric acid buffer. ●, 2.0 ml. of 0.01 M buffer; O, 1.0 ml. of 0.02 M buffer and 1.0 ml. of 36 per cent aqueous formaldehyde adjusted to pH 7.0.

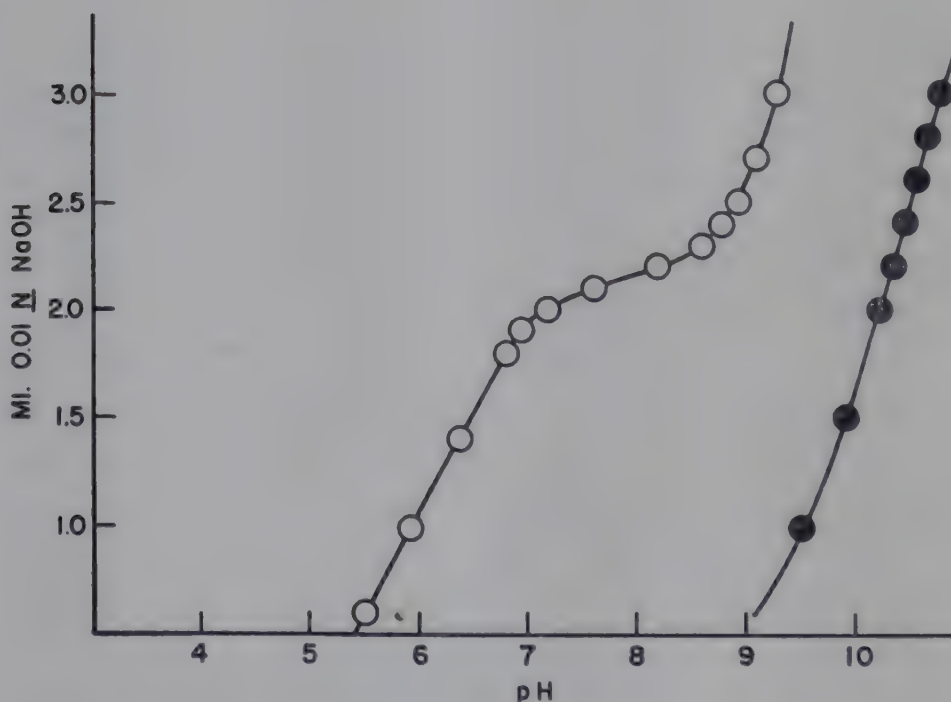


FIG. 2. Titration of ethylenediamine-hydrochloric acid buffer. ●, 2.0 ml. of 0.01 M buffer; O, 1.0 ml. of 0.02 M buffer and 1.0 ml. of 36 per cent aqueous formaldehyde adjusted to pH 7.0.

enzyme, and hydrolysis products. The effect of the hydrolysis products, either singly or in combination, upon the end-point of the titration is illustrated by the titration curves given in Fig. 3. The data of Fig. 4 clearly

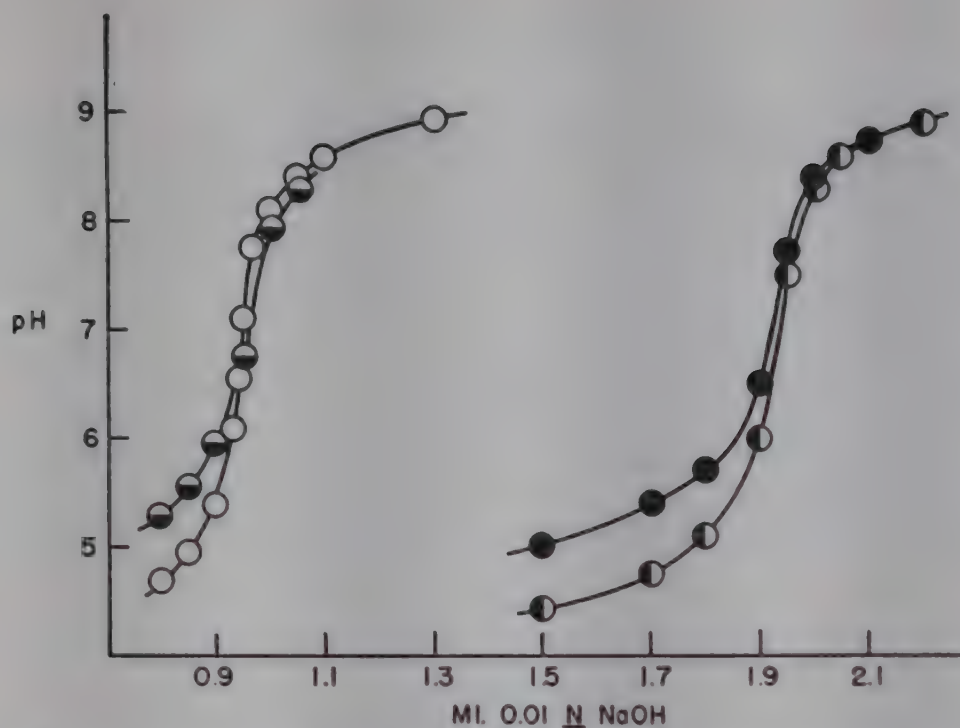


FIG. 3. Formol titration of tris(hydroxymethyl)aminomethane-hydrochloric acid buffer and of buffer and hydrolysis products. \circ , 1.0 ml. of 0.02 M buffer and 1.0 ml. of 36 per cent aqueous formaldehyde adjusted to pH 7.0; \ominus , 1.0 ml. of solution 0.02 M in buffer and 0.01 M in ammonia and 1.0 ml. of formaldehyde solution; \bullet , 1.0 ml. of solution 0.02 M in buffer and 0.01 M in acetyl-DL-phenylalanine and 1.0 ml. of formaldehyde solution; \bullet , 1.0 ml. of solution 0.02 M in buffer, 0.01 M in ammonia, 0.01 M in acetyl-DL-phenylalanine, and 1.0 ml. of formaldehyde solution.

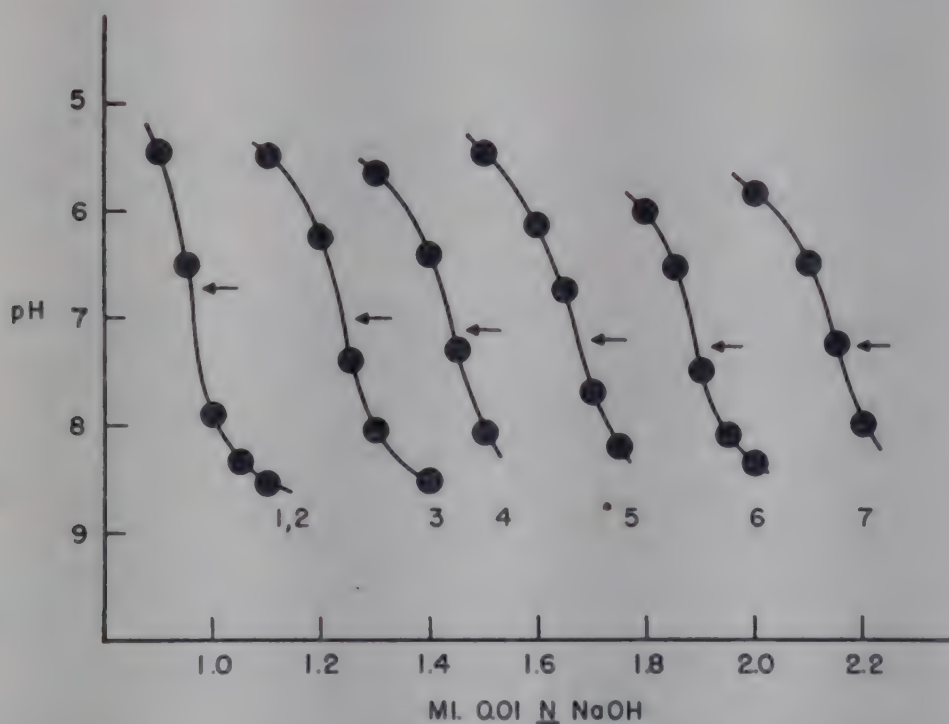


FIG. 4. Formol titration curves obtained during hydrolysis of nicotinyl-L-tryptophanamide by chymotrypsin at pH 7.8 and 25°. Curve 1, buffer; Curve 2, buffer plus substrate; Curve 3, buffer plus substrate plus enzyme, $t = 1$ minute; Curve 4, same as Curve 3 except $t = 10$ minutes; Curve 5, same as 3 except $t = 20$ minutes; Curve 6, same as Curve 3 except $t = 30$ minutes; Curve 7, same as Curve 3 except $t = 60$ minutes. End-point indicated by arrow.

show that every component in an enzymatic digest, with the exception of neutral substrates which do not react with formaldehyde, can influence the end-point of the formol titration. Curves 1 and 2, which are superimposable, are formol titration curves of a 0.02 M tris(hydroxymethyl)aminomethane-hydrochloric acid buffer of pH 7.8 and of this buffer plus 10 μ M of nicotinyl-L-tryptophanamide per ml. of solution respectively. Curve 3 is the formol titration curve of a solution 0.02 M in buffer containing 10 μ M of substrate and an amount of chymotrypsin equivalent to 0.15 mg. of protein nitrogen per ml. of solution determined 1 minute after the addition of the enzyme. Curves 4 to 7 are similar titration curves determined 10, 20,

TABLE I

*Hydrolysis of Nicotinyl-L-tryptophanamide by Chymotrypsin in Presence of Various Buffers**

Time	Per cent hydrolysis						
	NH ₂ CH ₂ CH ₂ NH ₂ ·HCl			(CH ₂ OH) ₃ CNH ₂ ·HCl			Phosphate
	0.005 M	0.02 M	0.05 M	0.005 M	0.02 M	0.1 M	0.02 M
<i>min.</i>							
10	25	25	24	24	23	21	27
20	45	45	51	46	46	50	52
30	64	67	67	68	68	69	70
40	75	77	81	77	79	83	82
50	83	85	85	86	87	87	87
60	89	92	88	93	94	94	94

* At 25°, pH 7.8, initial substrate concentration $s_0 = 10 \mu$ M per ml. of reaction mixture, initial enzyme concentration $E_0 = 0.15$ mg. of protein nitrogen per ml. of reaction mixture.

30, and 60 minutes after the addition of the enzyme. With the end-point of the titration varying with the extent of hydrolysis, it may be concluded that in order to obtain maximum accuracy in the determination of proteolytic activity by the above method it is imperative to use a potentiometric titration (8) so that the end-point of the titration may be determined coincidentally with the extent of hydrolysis at any given time.

In any enzyme-catalyzed reaction it is necessary to demonstrate that the buffer used does not participate in the reaction other than to control the pH of the system if unambiguous results are to be obtained. Accordingly the chymotrypsin-catalyzed hydrolysis of nicotinyl-L-tryptophanamide was studied at pH 7.8 and 25° in three different buffer systems and at three different concentrations of two of the three buffers. These data are given in Table I. In a second series of experiments a similar study was made in respect to the hydrolysis of nicotinyl-L-phenylalaninamide, though in this

latter case only two buffers were compared at a single buffer concentration (Table II). The data given in Tables I and II clearly show that within the limits of experimental error the rates of hydrolysis of the two substrates above at pH 7.8 and 25° are essentially independent of the nature of the buffer, and it may be concluded that none of the three buffers participates in the hydrolytic reaction other than to control the pH of the system. In view of the fact that similar results have been obtained with a number of other specific chymotrypsin substrates² it would appear that either ethylenediamine or tris(hydroxymethyl)aminomethane buffers may be used without fear of complications in any chymotrypsin-catalyzed hydrolysis provided the buffer concentration is not allowed to exceed that required to insure adequate buffering of the system.

TABLE II

*Hydrolysis of Nicotinyl-L-phenylalaninamide by Chymotrypsin in Presence of Various Buffers**

Time <i>min.</i>	Per cent hydrolysis	
	NH ₂ CH ₂ CH ₂ NH ₂ ·HCl, 0.02 M	Phosphate, 0.02 M
15	21	20
30	40	38
60	63	62
90	79	78
120	92	90

* At 25°, pH 7.8, initial substrate concentration $s_0 = 10 \mu\text{M}$ per ml. of reaction mixture, initial enzyme concentration $E_0 = 0.15$ mg. of protein nitrogen per ml. of reaction mixture.

In Figs. 1 to 4 it will be noted that there is a large potential change in the region of the end-point when tris(hydroxymethyl)aminomethane is used as a buffer. While there is no doubt that coincidental determination of the end-point of the titration and the extent of hydrolysis by a potentiometric titration will give the most accurate results, it is obvious that in some cases an indicator titration could be used, though with some sacrifice in accuracy. The data given in Table III illustrate the point that, if the pH of the end-point is known and is invariant with respect to time, the precision of the indicator titration is of the same order of magnitude as that of the potentiometric titration. However, in the determination of proteolytic activity there is no guarantee that the end-point of the titration will be the same for all substrates, for all proteolytic enzyme preparations, and for all concentrations of these components. This fact and the

² Unpublished experiments of R. V. MacAllister, H. T. Huang, and B. M. Iselin.

fact that for any given initial substrate and enzyme concentration the pH of the end-point of the titration does vary with time clearly establish the limitations of the above indicator titration even though it is decidedly superior to those in which the titration is conducted in a highly buffered system (9-11).

TABLE III
*Titration of Simulated Reaction Mixture**

0.10 N NaOH consumed per 2.0 ml. mixture		
Potentiometric titration; end-point, pH 7.25	Phenol red titration; end-point, orange color	Brom thymol blue titration; end-point, blue color
ml.	ml.	ml.
1.94	1.97	1.94
1.96	1.95	1.96
1.96	1.96	1.93
1.95	1.93	1.95
1.95	1.95	1.96
1.95	1.96	1.97
1.95 \pm 0.01	1.95 \pm 0.02	1.95 \pm 0.02

* 1.0 ml. of a solution 0.02 M in tris(hydroxymethyl)aminomethane-hydrochloric acid buffer, 0.01 M in acetyl-DL-phenylalanine, and 0.01 M in ammonia and 1.0 ml. of 36 per cent aqueous formaldehyde, pH 7.0.

EXPERIMENTAL

Reagents—A 0.20 M stock solution of the tris(hydroxymethyl)aminomethane-hydrochloric acid buffer was prepared by dissolving 12.11 gm. of the amine (obtained from the Commercial Solvents Corporation), m.p. 168-169° after two recrystallizations from ethanol, in the minimum quantity of water, adding 50 ml. of 1 N hydrochloric acid, and making the solution up to 500 ml. The pH of this solution was 8.05 and that of a 0.02 M solution 7.85. A 0.20 M stock solution of the ethylenediamine-hydrochloric acid buffer was prepared from 6.01 gm. of freshly distilled ethylenediamine and 107 ml. of 1.0 N hydrochloric acid made up to 500 ml. The pH of this solution was 8.0 and that of a 0.02 M solution 7.80. The concentration of all buffers is given in respect to the amine component. The two substrates used in this study, *i.e.* nicotinyl-L-tryptophanamide and nicotinyl-L-phenylalaninamide, were prepared by the condensation of nicotinyl azide with the amino acid ester and subsequent ammonolysis (12). The chymotrypsin used was an Armour preparation.

Procedure—The desired quantity of substrate was weighed into a 10.0 ml. calibrated glass-stoppered volumetric flask, dissolved in 5 to 7 ml. of

hot water, 1.0 ml. of 0.20 M buffer solution added, the solution brought to thermal equilibrium, the desired amount of enzyme preparation added,³ the solution made up to volume, and thoroughly mixed. 1 ml. aliquots, withdrawn from the solution immediately after mixing and at subsequent selected time intervals, were added to 1.0 ml. of 35 per cent aqueous formaldehyde, previously adjusted to pH 7.0 by the addition of 0.1 N sodium hydroxide, and contained in a 20 mm. \times 50 mm. shell vial, and the mixture was immediately titrated potentiometrically with standard 0.01 N sodium hydroxide by use of a Beckman model G pH meter equipped with No. 270-6 calomel electrode and a No. 290-11 glass electrode. The semi-automatic burette, graduated in 0.01 ml., was equipped with a capillary tip of sufficient length to permit introduction of the reagent beneath the surface of the solution being titrated which was stirred by rotation of the shell vial (13). The end-point of the titration and extent of hydrolysis were determined by examination of the curve constructed for each titration. It should be pointed out that this procedure consumes no more time than that taken in trying to decide whether or not the end-point has been reached in an indicator type of titration and is far more objective. In all cases blank experiments in which enzyme or substrate was omitted were performed coincidentally.

Results

The data given in Figs. 1 to 3 are self-explanatory. The potentiometric formol titration curves given in Fig. 4 were obtained by the titration of 1.0 ml. aliquots of a system 0.02 M in tris(hydroxymethyl)aminomethane-hydrochloric acid buffer with an initial substrate concentration, s_0 , of 10 μ M per ml. of solution and an initial enzyme concentration, E_0 , of 0.15 mg. of protein nitrogen per ml. of solution after the addition of 1.0 ml. of 36 per cent formaldehyde previously adjusted to pH 7.0. The data given in Tables I and II were obtained as described above and those in Table III are self-explanatory.

SUMMARY

A procedure for the determination of proteolytic activity, based upon coincidental use of a primary or secondary amine buffer system and a potentiometric formol titration, has been described and it has been shown that in order to obtain accurate results the end-point of the titration must be determined coincidentally with the extent of hydrolysis. For studies with chymotrypsin, a tris(hydroxymethyl)aminomethane-hydrochloric acid buffer is recommended.

³ It is recommended that whenever possible the solid enzyme preparation be weighed out for each individual hydrolysis experiment and dissolved in about 1 ml. of water just prior to the addition to the system.

BIBLIOGRAPHY

1. French, D., and Edsall, J. T., *Advances in Protein Chem.*, **2**, 277 (1945).
2. Michaelis, L., *J. Biol. Chem.*, **87**, 33 (1930).
3. Michaelis, L., and Schubert, M. P., *J. Biol. Chem.*, **115**, 221 (1936).
4. Kirby, A. H. M., and Neuberger, A., *Biochem. J.*, **32**, 1146 (1938).
5. Leonis, J., *Compt.-rend. trav. Lab. Carlsberg, Série chim.*, **26**, 357 (1948).
6. Schwartzbach, G., *Helv. chim. acta.*, **16**, 522 (1933).
7. Gomori, G., *Proc. Soc. Exp. Biol. and Med.*, **62**, 33 (1946).
8. Dunn, M. S., and Loshakoff, A., *J. Biol. Chem.*, **113**, 359 (1936).
9. Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, **183**, 32 (1929).
10. Willstätter, R., and Persiel, H., *Z. physiol. Chem.*, **142**, 245 (1925).
11. Willstätter, R., Waldschmidt-Leitz, E., Dunaiturria, S., and Kustner, G., *Z. physiol. Chem.*, **161**, 191 (1926).
12. Iselin, B. M., Huang, H. T., MacAllister, R. V., and Niemann, C., *J. Am. Chem. Soc.*, in press.
13. Harmon, K. M., and Niemann, C., *J. Biol. Chem.*, **178**, 743 (1949).

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